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Electron donor system for enzymes and its use in the biochemical conversion of substrates

The invention relates to a novel electron donor system for enzymes with redox properties, and to the use thereof in enzyme-catalyzed oxidation reactions such as, in particular, the preparation of ω-hydroxylated fatty acids. The invention additionally relates to an improved detection method for fatty acid monooxygenases, to bioreactors and to test kits in which the electron donor system can advantageously be employed.

The biotechnological use of enzymes with redox properties, such as, for example, monooxygenases, in cell-free reaction systems is always associated with the problem that the use of natural cofactors for providing the necessary redox equivalents (such as, for example, NADH or NADPH) is associated with unacceptably high costs.

This also applies to the biotechnological use of cytochrome P450-containing monooxygenases. All P450 enzymes have the common function of transferring oxygen atoms to unactivated aliphatic or aromatic X-H (X = -C, -N, -S) bonds. In addition, P450 enzymes are able to epoxidize the -C=C- double bond. Most P450 systems require for these oxygenation reactions cofactors such as NADPH or NADH as source of electrons. P450 systems are divided into four classes in accordance with the implementation of this electron transfer system (reductase system). Class I P450 enzymes contain as reductase an FAD domain and another Fe-S protein (usually mitochondrial and bacterial P450 enzymes), classe II P450 enzymes have an FAD/FMN reductase (usually ER P450 enzymes) and class III P450 enzymes require no other reducing equivalents; they convert peroxygenated substrates which already contain oxygen. The only P450 enzyme of class IV obtains its electrons directly without a transfer system from NADH.

The functional diversity of the monooxygenations catalyzed by P450 systems of compounds which can often be obtained by chemical means only with difficulty offers an enormous biotechnological, pharmacological and toxicological potential. One important precondition for *in vitro* use of these potentials is the development of suitable expression, purification and, in particular, activity detection systems which permit P450 systems to be characterized and enzyme variants with "improved" properties to be found.

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A further important precondition for the use of these P450 monooxygenases, in particular of classes I and II, would be the availability of an electron donor system at reasonable cost.

An NADPH cofactor recycling system has been described by Deffner et al., Ann. N.Y. Acad. Sci.(1987) 501, 171. However, on use on the preparative scale, this represents a cost problem and makes reactions carried out in enzyme-membrane reactors more complicated. Alternative electron donors have therefore been sought. One promising route is the electrochemical reduction of P450 enzymes. Estabrook et al.(Methods Enzymol. (1996) 272, 44) were able to determine conversions for six different P450 enzymes using a Co(III) sepulchrate mediator system and Pt electrodes. The activities were, however, about 8-times less than with the reducing equivalent NADPH. In place of the mediator, sodium dithionite (Fang et al., Drug. Metab. Dispos.(1996) 24(11): 1282) was found to be a compound which directly reduces P450 enzymes. In the case of P450 BM-3, the activity was reduced with sodium dithionite by a factor 8150 compared with NADPH; it is presumed that P450 BM-3 is triply reduced by sodium dithionite and thus mostly inactivated.

It is an object of the present invention firstly to provide an efficient, alternative electron donor system at reasonable cost for enzymes with redox properties. It was particularly intended that this system be usable in biochemical conversions in which cytochrome P450-containing enzymes are involved. It was additionally intended to provide an improved detection method for P450 enzymes. A further object of the invention was to provide an improved biotechnological method for the enzymatic transfer of oxygen to organic molecules and, in particular, for the terminal or subterminal hydroxylation of hydrocarbyl compounds having a polar group, such as in particular fatty acids.

We have found that these objects are achieved by providing an electron donor system for transferring electrons to enzymes with redox properties, wherein the system comprises an inorganic, non-electrode-bound source of electrons and a mediator which is able to transfer electrons from the source of electrons to the enzyme. It is possible in this connection for the components of the system to be present singly or in a mixture, in carrier-bound or, preferably, non-carrier-bound form.

A "non-electrode-bound source of electrons" for the purpose of the invention is able to deliver electrons to the mediator without application of an external voltage between a pair of

electrodes. This entails in particular reduction of the mediator by the source of electrons via a chemical redox reaction.

The electron donor system provided according to the invention can be used in particular for cytochrome P450-containing enzymes such as, for example, the enzymes comprised in the large family of monooxygenases (E.C. 1.14.-.-). A nonlimiting example which may be mentioned here is the cytochrome P450 monooxygenase BM-3 which can be isolated from Bacillus megaterium.

In a preferred embodiment of the electron donor system, the latter comprises a mediator with a standard normal potential (for example measured against a calomel electrode; under standard conditions; cf. Creasar et al. below) in the region of less than about -0.4 V. Nonlimiting examples of mediators which may be used according to the invention are cobalt(III) sepulchrate, methylviologen, neutral red, riboflavin, ruthenium triacetate, FMN and FAD, with cobalt(III) sepulchrate being preferred. Cobalt(III) sepulchrate has, for example under standard conditions, a normal potential of -0.54 V (cf. Creasar et al. (1977), J. Am. Chem. Soc., 99, 3181).

The electron donor system according to the invention preferably comprises as source of electrons a metal with a lower standard normal potential than the mediator. A nonlimiting example of the source of electrons which may be mentioned here is metallic zinc. The metal is for this purpose preferably present in a reactive form, i.e. with a large surface area, such as, for example, in powder form.

Preferred electron donor systems according to the invention comprise the following combinations of source of electrons and mediator:

- Zn/cobalt(III) sepulchrate or
- Zn/neutral red.

The invention further relates to a method for the enzymatic oxidation of, i.e. transfer of oxygen to, a hydrocarbon-containing hydrogen donor molecule (i.e. an oxidizable compound), which comprises incubating the hydrogen donor molecule in a reaction medium comprising the oxygen-transferring enzyme and an electron donor system complying with the above definition, in the presence of oxygen under reaction conditions.

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An "oxidation" for the purpose of the present invention relates to enzyme-catalyzed oxidation reactions in the widest sense. In particular, the term comprises oxidation reactions which are catalyzed by enzymes from the class of monooxygenases (E.C.1.14.-.-) and, in particular, cytochrome P450 enzymes. These oxidation reactions comprise:

a) carbon oxidations at saturated or unsaturated, aliphatic or aromatic carbon atoms, e.g. hydroxylations and epoxidations;
b) sulfur oxidations;
c) nitrogen oxidations;
d) oxidative dealkylations; and
e) oxidative dehalogenations.

The hydrogen donor molecule is preferably selected from compounds of the formula

R-X

in which

R is a straight-chain or branched, preferably straight-chain, alkyl radical with 8 or more carbon atoms, such as, for example, 10 to 30 carbon atoms, and

X is a polar group capable of forming hydrogen bonds, preferably a carboxyl, amide, nitrile, sulfate, sulfone, amine or hydroxyl group.

A preferred variant of the above method relates to the enzymatic preparation of terminally or subterminally (i.e. in position ω-1 to ω-4) hydroxylated fatty acids, which comprises

a) converting a hydroxylatable fatty acid or a hydroxylatable fatty acid derivative in the presence of an electron donor system complying with the above definition using a cytochrome P450 monooxygenase and oxygen; and

b) isolating the hydroxylated product(s).

The ω-hydroxylatable (i.e. terminally or subterminally hydroxylatable) fatty acid or its derivative is according to the invention preferably selected from or derived from terminally saturated, branched or unbranched fatty acids with 8 or more, e.g. more than 10 carbon atoms, in particular C_{12} - C_{30} fatty acids.

Nonlimiting examples of hydroxylatable fatty acids according to the invention which should be mentioned are: caprylic, pelargonic, undecanoic, lauric, tridecanoic, myristic, pentadecanoic, palmitic, margaric, stearic, nonadecanoic, arachic, behenic, lignoceric, cerotic and melissic acids.

Examples of suitable fatty acid derivatives are C_1-C_4-alkyl esters, amides or anhydrides with, preferably, short-chain, in particular C_1-C_4-carboxylic acids.

The enzyme preferably used in the methods according to the invention described above is a cytochrome P450 monooxygenase selected from:

- cytochrome P450 monooxygenases of the enzyme class E.C. 1.14.-.-, in particular from the CYP102 P450 family, or of eukaryotic or prokaryotic, in particular bacterial, origin, and preferably the wild-type enzyme which can be isolated from Bacillus megaterium (DSM 32T); or
- a mutant thereof.

A preferred group of mutants is derived from cytochrome P450 monooxygenase BM-3 from *Bacillus megaterium* with an amino acid sequence as shown in SEQ ID NO: 35, which has at least one functional mutation in one of the following amino acid sequence regions: 24-28, 45-51, 70-72, 73-82 (helix 5), 86-88 (helix 6), 172-224 (F/G loop) and 352-356 (ß strand 8); and functional equivalents of these mutants.

A "functional mutation" for the purpose of the present invention comprises an amino acid replacement in the stated sequence regions, which leads to a mutant which is still able to catalyze one of the abovementioned oxidation reactions such as, for example, hydroxylations.

Particularly preferred according to the invention are P450 BM-3 monooxygenase mutants, each of which comprise at least one functional mutation in the amino acid sequence regions 86-88, 172-224, 73-82, 45-51, 24-28, 70-72 and 352-356 (as shown in SEQ ID NO: 35), singly or in combination.

It is possible in particular for an amino acid substitution to be present in at least one of positions 26, 47, 72, 74, 87, 188 and 354 as shown in SEQ ID NO: 35 of the wild-type enzyme.

Thus, for example, Phe87 can be replaced by an amino acid with an aliphatic side chain, such as, for example, Ala, Val, Leu, in particular Val or Ala; Leu188 can be replaced by an amino acid

with an amine or amide side chain, such as, for example, Asn, Gln, Arg or, in particular, Lys, or amino acids such as Ala, Gly, Ser and Trp; Ala74 can be replaced by another amino acid with an aliphatic side chain, such as, for example, Val and, in particular, Gly; Arg47 can be replaced by an amino acid with a cyclic side group, such as, for example, His, Tyr or, in particular, Phe; and Val26 can be replaced by an amino acid with a hydroxyl side group, such as, for example, Ser or, in particular, Thr. For example, Ser72 can be replaced by an amino acid with an aliphatic side chain, such as, for example, Ala, Val, Leu, Ile and, in particular, Gly, and Met354 can be replaced by an amino acid with a hydroxyl side group, such as, for example, Ser or, in particular, Thr.

Examples of mutants are:

a) F87V;
b) F87A, L188K;
c) F87V, L188K;
d) F87A, L188K; A74G;
e) F87V, L188K, A74G;
f) F87A, L188K, A74G, R47F;
g) F87V, L188K, A74G, R47F;
h) F87A, L188K, A74G, R47F, V26T; or
i) F87V, L188K, A74G, R47F, V26T;
k) V26T,
l) R47F,
m) S72G,
n) A74G,
o) F87A
p) L188z, in which z is K, R, W, Q, N, G, A or S, and
q) M354T;

and functional equivalents thereof.

Suitable mutants are also described, for example, in the earlier DE-A-100 11 723, which is incorporated herein by reference.

The mutants and functional equivalents employed may moreover show an "altered substrate profile" by comparison with the wild-type enzyme.

An "altered substrate profile" means for the purpose of the present invention in particular:

a) a change in the reactivity, such as, for example, an increase in the specific activity (expressed as nmol of substrate converted/minute/nmol of P450 enzyme) and/or at least one kinetic parameter selected from Kcat, Km and Kcat/Km, for example by at

least 1%, such as, for example, 10 to 1000%, 10 to 500%, or 10 to 100%, of the mutant for at least one oxidizable substrate compared with the wild-type enzyme; and/or

b) a change in the regioselectivity, such as, for example, a shift of the preferred position for the oxidation reaction; this may entail, for example, a shift of the preferred terminal or subterminal (ω-1, ω-2, ω-3, ω-4, in particular ω-1 to ω-3) hydroxylation position on at least one hydroxylatable carboxylic acid or one hydroxylatable derivative of an aliphatic carboxylic acid. The alteration of the substrate profile may moreover be expressed over the entire size range (i.e. C_8-C_{30}) or only in part-regions, for example for C_8-C_{12}-, C_{10}-C_{12}-, C_{12}-C_{30}-, C_{12}-C_{25}- or C_{12}-C_{20} carboxylic acids or for individual carboxylic acids from these part-ranges.

"Functional equivalents" also mean according to the invention mutants which have in at least one of the abovementioned sequence positions an amino acid substitution other than that specifically mentioned but still lead to a mutant which, just like the specifically mentioned mutant, catalyzes one of the oxidation reactions defined above.

Functional equivalence also exists in particular when the alterations in the reactivity pattern are in qualitative agreement.

"Functional equivalents" also include, of course, P450 monooxygenase mutants which are obtainable in the same way as the specifically mentioned P450 BM3 mutants by mutation of P450 enzymes from other organisms. For example, it is possible by comparing sequences to establish ranges of homologous sequence regions. It is then possible, with the modern methods of molecular modeling, to undertake equivalent mutations influencing the reaction pattern based on the specific requirements of the invention.

"Functional equivalents" likewise include the mutants obtainable by one or more additional amino acid additions, substitutions, deletions and/or inversions, it being possible for said additional changes to occur in any sequence position as long as they lead to a mutant with an "altered substrate profile" in the above sense.

Mutants which are preferably employed comprise a mutation in position 87 and are selected from the mutations F87A or F87V, which optionally has at least one other of the following mutations: L188K, A74G, R47F and V26T. Particular preference is

given to the one-fold mutant F87A and the five-fold mutant F87A, L188K, A74G, R47F, V26T (amino acids indicated in the one-letter code; original amino acid to the left of the stated position; new amino acid to the right of the stated position).

The above methods are preferably carried out using the zinc/Co(III) sepulchrate electron donor system.

In a preferred embodiment of the method, the conversion was carried out in the presence of chloride ions.

The presence of a hydrogen peroxide-cleaving enzyme, e.g. catalase, may also be advantageous.

The invention further relates to a bioreactor for use for the enzymatic transfer of oxygen to a hydrocarbon-containing hydrogen donor molecule and, in particular, to the use for producing ω-hydroxylated fatty acids, which comprises an immobilized monooxygenase (e.g. immobilized on a carrier material, e.g. DEAE 650M or Super Q650M) and an electron donor system complying with the above definition in a liquid reaction medium. The specific design of the reactor is based on conventional expert knowledge.

The invention further relates to a detection method for fatty acid monooxygenases, which comprises

a) incubating an analyte suspected of having enzymic activity with an ω-hydroxylatable fatty acid or fatty acid derivative which has a terminal fluorophore or chromophore which can be eliminated, in the presence of an electron donor system complying with the above definition; and

b) determining the elimination of the fluorophore or chromophore qualitatively or quantitatively.

A suitable analyte is, for example, a cell homogenate, e.g. of a bacterial strain. It is possible to screen for example collections of strains for enzyme activity using the detection method according to the invention.

The reaction in this case is preferably carried out in the presence of a hydrogen peroxide-cleaving enzyme and, where appropriate, in the presence of chloride ions.

Examples of suitable fluorophores and chromophores are phenols, catechols, hydroxycoumarins such as umbelliferone, phenoxazines and, in particular, resorufin.

Setting of the optimal parameters for the methods for the enzymatic conversions and detection reactions described above is directly possible by taking account of the instructions in the following experimental section. Thus, for example, without being restricted thereto, a cobalt(III) sepulchrate concentration of more than 0.1 mM, such as, for example, 0.5 to 1.0 mM, is particularly advantageous; likewise a pH of about 8 to 8.5; a zinc concentration of more than about 2 mg/ml, such as, for example, about 5 to 50 mg/ml. The hydrogen peroxide-cleaving enzyme can be employed for example in amounts of about 10 to 5000 U/ml.

Finally, the invention further relates to a test kit, in particular for carrying out the above detection method for carboxylic acid monooxygenases, comprising an electron donor system complying with the above definition.

The diverse possible uses of the present invention are explained further in the following sections.

<u>Catalyzed reactions of P450 systems and examples of industrial applications in fine chemicals synthesis</u>

In organisms, cytochrome P450 enzymes serve inter alia for ergosterol synthesis, the biosynthesis of insect hormones and phytohormones, the development of ripeness in fruit, odor and color in plants, the biosynthesis of glucocorticoids and mineralocorticoids, the aromatization of androgens to estrogens, the metabolism of retinoids to regulate normal and epithelial growth/differentiation, arachidonic acid metabolism to form prostaglandins, leukotrienes and thromboxanes and the formation of vasoactive products. In addition, they serve to activate and detoxify xenobiotic substances.

P450 enzymes belong to the so-called phase I enzymes which prepare, by oxygenations, compounds which are insoluble in water or of low solubility for further metabolization. Phase II enzymes serve for this purpose, such as glutathione transferases, N-acetyltransferase or sulfotransferase, which add a polar group onto the former hydroxylated or epoxidized compounds. This makes these metabolites soluble in water and thus bioavailable. The diversity of reactions catalyzed by cytochrome P450 enzymes are summarized below and discussed in relation to their economic and

functional importance. Table 1 shows a summary, broken down into selected families.

Table 1

P450 family	Reactions of P450 enzymes
CYP1-3	Metabolization of xenobiotic substances; CYP1: PCB, PAH, dioxins, aflatoxins; CYP2: plant toxins, pesticides; CYP3: cyclosporins, erythromycin, also involved in steroid metabolism
CYP4, CYP52, CYP102	CYP4, CYP52, CYP102: terminal and/or subterminal oxidation of fatty acids CYP4: oxidation of eicosanoids (prostaglandins, leukotrienes and thromboxanes)
CYP11, CYP17, CYP21	Biosynthesis of gluco- and mineralocorticoids; CYP11: 11ß-hydroxylases, hydroxylation of deoxycorticosterone or deoxycortisol; CYP17: steroid 17α-hydroxylase; CYP21: steroid 21-hydroxylase
CYP19	Conversion of steroids into estrogens; aromatase
CYP27	Cholesterol 27-hydroxylase

The P450 enzymes of the CYP1-3 enzyme families are mainly responsible for the metabolization of xenobiotic substances; the oxidative reactions occurring therein are divided up hereinafter into C, S and N oxidations, dealkylation and dehalogenation.

a) C oxidations

P450 enzymes hydroxylate or epoxidize unactivated C-H bonds and/or C=C double bonds in aliphatic and aromatic systems. The metabolites which are formed are frequently toxic, such as the neurotoxin 2,5-hexanedione, or carcinogenic such as DNA-alkylating epoxides (e.g. styrene oxide). In addition, nitrosamines and benzopyrenes C-oxidized by P450 enzymes have carcinogenic effects.

Drugs such as barbiturates and phenobarbitals are likewise converted by P450 enzymes into their active form. The hydroxylation of unactivated -C-H bonds is one of the most useful biotransformation reactions, because competition from chemical methods is rare. In contrast to free-radical reactions, biohydroxylation reactions on C atoms show the following activity sequence: secondary> tertiary> primary. Well-known methods carried out industrially are the hydroxylation of progesterone at position 11α by *Aspergillus niger*, which made about half of the

37 conventional steps unnecessary, and the 7ß-hydroxylation of 3α-hydroxy-5ß-cholic acid by *Fusarium equiseti*. The product ursodeoxycholic acid is able to dissolve cholesterol and is employed in the treatment of gallstones. In asymmetric synthesis, biotransformations are used inter alia for synthesizing ß-hydroxybutyric acid, a starting material for vitamin syntheses (α-tocopherol), flavorings, antibiotics (calcimycin) and chiral branched epoxidized alkenes.

b) N-Oxidation / S-oxidation

P450 enzymes hydroxylate arylamines and acetylated amines, such as benzidine, 4-biphenylamine and 2-acetaminofluorene and comparable heterocycles produced on charring of foodstuffs, on the N atom. Further acetylation or sulfonation of the hydroxyl groups produces electrophilic substances with high carcinogenic activity.

Aryl and alkyl thioethers are oxidized by P450 monooxygenases directly on the S atom to sulfoxides. However, the oxidation frequently does not stop at the sulfoxide stage; many sulfoxides are oxidized further to the corresponding sulfone compounds, which often have cytotoxic effects. Microbial oxidations of chiral dithioacetals take place with a high enantiomeric excess using *Corynebacterium equi* and *Helminthosporium sp*.

c) Oxidative dealkylations

P450 enzymes also catalyze oxidative O-, N- and S-dealkylation. Examples of these important reactions are the O-dealkylation of codeine, mescaline, phenacetin, the N-dealkylation of ephedrine, methamphetamine, aminopurine and the S-dealkylation of 6-methylthiopurine to 6-methylpurine.

d) Oxidative deamination

P450 enzymes also catalyze the oxidative deamination of amines. This reaction mainly has a detoxifying function for the organism. Histamine, norepinephrine and mescaline inter alia can be deaminated in this way.

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e) Oxidative dehalogenation

Many halogenated alkanes and alkenes form after hydroxylation at a -C-H bond an unstable intermediate which dissociates into an aldehyde or ketone and H-Hal. Examples are the oxidation of ethylene dibromide to 2-bromoacetaldehyde and the oxidation of chloroform to phosgene. Mono- and dihalogenated methane forms metabolites whose cytotoxicity is only low compared with the trihalogenated chloroform.

A mechanistic description of the types of P450 reactions which have been presented is to be found in the review article by Koymans et al., in Xenobiotica,(1993) 23(6):633.

<u>In vivo / *in vitro* use of P450 monooxygenases for the synthesis of fine chemicals:</u>

In contrast to microbial biotransformation, the use of recombinantly expressed P450 monooxygenases for synthesizing fine chemicals is confined, despite an enormous potential, to a few *in vivo* methods such as the preparation of dicarboxylic acids as intermediates for odorants, steroids such as progesterone and subterminally hydroxylated saturated and unsaturated fatty acids as precursors of fragrances (lactones), polymers and dosage forms for medicines.

The use of P450 monooxygenase enzymes *in vitro* for the synthesis of fine chemicals has not to date been disclosed in the literature. In order to use P450 monooxygenases for synthesizing fine chemicals, it is necessary for expression systems to be available which permit functionally active P450 to be expressed in high yields beyond species boundaries. In addition, the enzyme must have sufficiently high activity and stability (temperature stability, solvent stability, oxidation stability). The produced metabolites ought not to be toxic for the protein and the host. For class I and class II in addition a suitable cofactor recycling system ought to be available. The present invention creates the conditions for this.

<u>The P450 BM-3 monooxygenase system</u>

P450 BM-3 (CYP102) was expressed with functional activity in *E. coli*, and characterized, for the first time in 1986 as third P450 monooxygenase from *Bacillus megaterium*. With a molecular weight of 118 kDa, P450 BM-3 was the first known water-soluble natural fusion protein which contains all three domains (FAD, FMN, P450) in a single polypeptide chain. Crystallization of the P450 domain

without substrates was achieved in 1993 (Ravichandran et al., Science (1993) 261, 731), and with substrate was achieved in 1997 (Li et al. Nature Structural Biology (1997), 4(2): 140). P450 BM-3 serves, because of its sequence homology to eukaryotic P450s and with the lack of eukaryotic P450 crystal structures, as preferred structural model for these P450 enzymes.

P450 BM-3 is a fatty acid hydroxylase which subterminally hydroxylates carboxylic acids, alcohols, amides, alkylammonium compounds from chain length C12 to C22. The regiospecificity of the hydroxylation depends, as shown in Table 2, greatly on the chain length of the fatty acid.

Table 2

Substrates	ω-Hydroxy-lation [%]	ω-1 Hydroxy-lation [%]	ω-2 Hydroxy-lation [%]	ω-3 Hydroxy-lation [%]
Lauric acid (12:0)	-	**48**	26	26
Myristic acid (14:0)	-	**58**	21	21
Palmitic acid (16:0)	-	20	**48**	31

The invention is now explained in more detail with reference to the appended figures and to the following examples which relate to the use of the donor system according to the invention to the hydroxylation of fatty acids using P450 enzymes. In this connection

Figure 1 shows the P450 BM3 cloning scheme for the example of pCYTEXP1;

Figur 2 shows the principle of the spectroscopic P450 detection according to the invention;

Figure 3 shows the absorption spectrum for the reduction of cobalt(III) sepulchrate with Zn powder in Tris/HCl, 0.2 M, pH 8.2; dotted line Co(III) sepulchrate oxidized; full line Zn/Co(III) sepulchrate reduced;

Figure 4 shows the change in absorption on conversion of 12-pNCA by P450 BM-3 F87A with Zn/Co(III) sepulchrate in Tris/HCl, 50 mM, pH 8.2, 0.25 M KCl;

Figure 5 shows an outline of the electron transfer route from Zn/Co(III) sepulchrate to P450 with shunt reaction pathway and pNCA as substrate;

Figure 6 shows the conversion of 12-PCA by P450 BM-3 F87A and Zn/Co(III) sepulchrate in Tris/HCl, 50 mM, pH 8.2, 0.25 M KCl with and without addition of catalase;

Figure 7 shows the effect of the Tris/HCl and potassium phosphate buffer mixtures on the reduction of p-nitrophenolate in the presence of P450 BM-3 F87A and Zn/Co(III) sepulchrate;

Figure 8 shows (A) 12-pNCA conversion by P450 BM-3 F87A as a function of the Co(III) sepulchrate concentration; (B) the effect of Co(III) sepulchrate on the activity of P450 BM-3 F87A in the presence of substrate;

Figure 9 shows the 12-pNCA conversion as a function of the amount of zinc powder employed in the presence of 0.5 mM Co(III) sepulchrate;

Figure 10 shows the remaining P450 BM-3 F87A activity after incubation with various concentrations of hydrogen peroxide without substrate for 5 min.

Reference Example 1: Microbiological methods

1. Microorganisms and plasmids, chemicals and enzymes

The bacterium *Bacillus megaterium* was purchased from the DSMZ collection of strains (32^T, Braunschweig, Germany), the *E.coli* strains DH5α, JM105 and JM109 were purchased from Clontech (Heidelberg, Germany) and XL1-Blue was purchased from Stratagene (Heidelberg, Germany). The strain W3110 was obtained from the Institut für Bioverfahrenstechnik (Stuttgart, Germany). The *E.coli* strain DH5α (supE44, lacU169 [80lacZ M15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was, unless otherwise mentioned, used for the cloning work described below. The plasmids used were pCYTEXP1 with the temperature-inducible P_RP_L promoter system of bacteriophage λ (Belev T.N., et al., Plasmid (1991) 26:147) and pASK-IBA1CA with tetracycline promoter (Schmidt, T.G.M., et al., J. Chromatogr. A (1994) 676:337; Schmidt, T.G.M. et al., J.Mol.Biol. (1996) 255:753). The strains and plasmids used in this work for heterologous expression of P450 BM-3 are summarized in Table 3, where pT indicates the origin of the P450 BM-3

constructs from the pCYTEXP1 plasmid and pA indicates the origin of the pASK-IBA1CA plasmid.

Table 3

E.coli strain/plasmid insert construct	expressed protein
DH5α/pT-USC0BM3,W3110/pT-USC0BM3	P450 BM-3
DH5α/pT-USC1BM3, JM105/pT-USC1BM3, JM109/pT-USC1BM3, W3110/pT-USC1BM3	P450 BM-3His_6
DH5α/pT-USC1BM3F87A, JM105/pT-USC1BM3F87A, JM109/pT-USC1BM3F87A, W3110/pT-USC1BM3F87A XL1-Blue/pT-USC1BM3F87A	P450 BM-3His_6 F87A Mutant
DH5α/pT-USC2BM3, W3110/pT-USC2BM3	P450 BM-3Glu_6
DH5α/pT-USC3BM3, W3110/pT-USC3MB3	P450 BM-3Arg_6
DH5α/pA-USC4BM3, JM105/pA-USC4BM3, JM109/pA-USC4BM3, W3110/pA-USC4BM3	P450 BM-3Strep

Unless otherwise mentioned, all the chemicals were purchased from Fluka Chemie (Buchs, Switzerland) and all the enzymes were purchased from New England Biolabs (Beverly, USA) or Boehringer Mannheim (Penzberg, Germany).

2. Culture media

All the complex media originated from Difco (Augsburg, Germany).

a) Nutrient medium (complete medium for *Bacillus megaterium*)

Peptone	5.0 g
Bacto beef	3.0 g
Bacto peptone	5.0 g
Agar	15 g (only for plate culture)
$MnSO_4*H_2O$	10 mg
H_2O	ad 1000 ml

Glycerol culture

Glycerol (86%)	500 µl
Culture(OD_{578}= 1.0)	500 µl

b) *Luria-Bertani*-Amp (LB-Amp, complete medium for E. coli)

Tryptone	10.0 g
NaCl	5.0 g
Yeast extract	5.0 g
Agar	20.0 g (only for plate culture)
Ampicillin	100 g/ml
H_2O	ad 1000 ml

3. Cultivation of the *E. coli* strains and of *Bacillus megaterium*

a) *Shaken flask experiments*

Bacillus megaterium was cultivated in the nutrient medium at 30°C, and the recombinant *E. coli* strains (DH5α, JM105, JM109, XL1-Blue, W3110) were cultivated in LB-Amp medium at 37°C. For this purpose, in each case one colony was transferred by an inoculating loop from an agar plate into 5 ml of LB-Amp. After cultivation at 30°C (*Bacillus megaterium*) and 37°C (*E. coli*) shaking at a rate of 220 rpm for about 18 h, 400 ml of medium in a 2 l flask were inoculated with 4 ml of culture. Induction of P450 expression in *E. coli* took place after the OD_{578} had reached between 0.8 and 1.0 by a heat-shock induction at 42°C for three to four hours in the case of the pT plasmid constructs or by adding anhydrotetracycline (final concentration 0.4 mg/l) in the case of the pA-USC4BM3 plasmid and incubating at 30°C for 14 hours.

b) *30-l batch fermentations*

The expression cassette pT-USC1BM3, the host DH5α and a bioengineering fermenter type LP351 were employed, with aeration at 3.5 l/min, a stirring speed of 300 rpm at 37°C in LB-Amp medium with an initial pH of 7.5, for preparative P450 BM-3 production. In order to avoid foaming and to increase yields, 2.0 ml of sterile Contraspum 210 (ZSchimmer & Schwarz, Lahnstein, Germany) and 0.1 mg/l $FeCl_3$ were added before inoculating the LB-Amp medium (Nishihara et al. 1997). Inoculation took place by adding two 400 ml shaken flask cultures (OD_{578}= 0.8-1.0; section a)). After the OD_{578} in the fermenter reached 1.0, P450 production took place by heat-shock induction by increasing the temperature from 37°C to 42°C for three to five hours. P450 BM-3 or the mutant F87A was expressed in *E. coli* DH5α in yields of from 36 to 48 mg/l of fermenter broth. The *E. coli* culture was concentrated from 30 liters to 2 liters by crossflow filtration using a Millipore application (Eschborn, Germany) with a Filtron (Dreieich, Germany) Centrasette OMEGA (0.3 μm) membrane. After centrifugation at 9200 g for 20 min, the cells were resuspended in potassium phosphate buffer (50 mM, pH 7.5) or Tris/HCl buffer (50 mM, pH 7.5), aliquoted in twelve 50 ml Falcon tubes (Greiner) and frozen at -20°C until used further.

Reference Example 2: Synthetic oligonucleotides

All the primers were prepared by ARK Scientific GmbH Biosystems (Dusseldorf, Germany). Only the primers for the point mutation and for isolating the P450 BM-3 gene from the genomic *Bacillus megaterium* DNA were purificed by HPLC.

a) *Oligonucleotides for genomic Bacillus megaterium DNA*

B1) 5'-GTGAAAGAGGGATCCCATGACAATTAAAGAAATGCC-3'
(SEQ ID NO: 1)

B2) 5'-GCCTCTTGGATCCTTACCCAGCCCACACGTCTTTTGCG-3'
(SEQ ID NO: 2)

b) *Primers for sequencing*

R0_5	5'-GTACGTGATTTTGCAGGAG-3'	(SEQ ID NO: 3)
R1	5'-GGCTATCATGCGATGATGGT-3'	(SEQ ID NO: 4)
R1_5	5'-CCCAGCTTATGATGAAAAC-3'	(SEQ ID NO: 5)
R2	5'-GGAAAAGATCCAGAAACGGG-3'	(SEQ ID NO: 6)
R2_5	5'-GTCGGCATGGTCTTAAACG-3'	(SEQ ID NO: 7)
R3	5'-ATTCCTCAGCTTCACCGTGA-3'	(SEQ ID NO: 8)
R3_5	5'-CTTGGCGGTATTCCTTCAC-3'	(SEQ ID NO: 9)
R4	5'-ATTTGCACCGCAGGTCGCAA-3'	(SEQ ID NO: 10)
R4_5	5'-CTGGGCTACTACGTATC-3'	(SEQ ID NO: 11)
R5	5'-TTCAATTTGTCGACAGCGCC-3'	(SEQ ID NO: 12)
R5_5	5'-GAAGGAGATCATTTAGGTG-3'	(SEQ ID NO: 13)
R6	5'-TCGCGCAATGGCTGCTAAAA-3'	(SEQ ID NO: 14)
R6_5	5'-CGATTTCTTCATCACCTC-3'	(SEQ ID NO: 15)
R7	5'-CTGCCAAAAGACCCTGAAAC-3'	(SEQ ID NO: 16)
L1	5'-ACCATCATCGCATGATAGCC-3'	(SEQ ID NO: 17)
L2	5'-CCCGTTTCTGGATCTTTTCC-3'	(SEQ ID NO: 18)
L3	5'-TCACGGTGAAGCTGAGGAAT-3'	(SEQ ID NO: 19)
L4	5'-TTGCGACCTGCGGTGCAAAT-3'	(SEQ ID NO: 20)
L5	5'-GGCGCTGTCGACAAATTGAA-3'	(SEQ ID NO: 21)
L6	5'-TTTTAGCAGCCATTGCGCGA-3'	(SEQ ID NO: 22)
L7	5'-GTTTCAGGGTCTTTTGGCAG-3'	(SEQ ID NO: 23)

c) *Oligonucleotides for tags at the C terminus*

A1) 5'-GTGAAAGAGGGATCCCATGACAATTAAAGAAATGCC-3'
(SEQ ID NO: 24)

A2) 5'-CGGAATTCTTAACGACGACGACGACGACGCCCAGCCCACACG-3'
(SEQ ID NO: 25)

G1) 5'-GTGAAAGAGGGATCCCATGACAATTAAAGAAATGCC-3'
(SEQ ID NO: 26)

G2) 5'-CGGAATTCTTATTCTTCTTCTTCTTCTTCCCCAGCCCACACG-3'

(SEQ ID NO: 27)

H1) 5'-GTGAAAGAGGGATCCCATGACAATTAAAGAAATGCC-3'

(SEQ ID NO: 28)

H2) 5'-CGCAATTCTTAATGATGATGATGATGATGCCCAGCCCACACG-3'

(SEQ ID NO: 29)

S1) 5'-GTCTCAGCGTGAGACCCCCAGCCCACACGTCTTTTGCC-3'

(SEQ ID NO: 30)

S2) 5'-GTGAAAGAGGTCTCCAATGACAATTAAAGAAATGCC-3'

(SEQ ID NO: 31)

d) *Primers for P450 BM-3 F87A point mutants*

F87A1 5'-GCAGGAGACGGGTTGGCCACAAGCTGGACGCATG-3'

(SEQ ID NO: 32)

F87A2 5'-CATGCGTCCAGCTTGTGGCCAACCCGTCTCCTGC-3'

(SEQ ID NO: 33)

Reference Example 3: Genetic engineering methods

1. Isolation and precipitation of genomic *Bacillus* DNA and plasmid DNA from *E. coli*

To isolate the genomic DNA from *Bacillus megaterium* by phenol/chloroform extraction, the cell pellet from 200 ml of culture supernatant (OD_{578}= 1.2) was resuspended in 20 ml of lysozyme mix (18 mg of lysozyme, 50 mM EDTA, 50 mM NaCl, 30 mM Tris/HCl, pH 8.0) and shaken at 220 rpm and 37°C for 30 minutes. After addition of 2 ml of SDS (10% (m/v)), incubation was continued at 37°C and 220 rpm for 60 minutes. Cell detritus was removed by extraction three times with a chloroform/isoamyl alcohol mixture (Roth, Karlsruhe, Germany) and once with phenol. The genomic DNA is precipitated by adding 10% (v/v) 3 M NaAc, pH 4.8 and 60% (v/v) isopropanol, rolled up on a glass rod, transferred into a Corex tube and centrifuged at 32,570 g. After washing with 70% ethanol three times, the DNA is dried under "house vacuum" and dissolved in 3 ml of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5). The purity of the DNA was determined spectroscopically by forming the ratio of 260 nm to 280 nm as 2.2.

The plasmid DNA was isolated from *E. coli* using the QIAprep spin miniprep kit, which is based on alkaline lysis of the cells, exactly in accordance with the manufacturer's (Qiagen) information.

2. Polymerase chain reaction (PCR)

a) PCR for isolating the P450 BM-3 gene from genomic Bacillus megaterium DNA

Standard PCR protocol

Repetitions of the program step	Denaturation	DNA annealing	Elongation/DNA-synthesis
Once	95°C, 7 min		
30 times	95°C, 1.5 min	50°C, 1.5 min	72°C, 2 min
Once			72°C, 7 min

Standard mixture

8 µl of dNTP mix (200µM), 10 µl of Taq polymerase buffer (10 x) without $MgCl_2$, 8 µl of $MgCl_2$ (25mM), 1 µl of primer B1 (0.1 µM), 1 µl of primer B2 (0.1 µM), 1 µl of genomic *Bacillus megaterium* DNA, 2.5 U of Taq polymerase (MBI Fermentas, Vilnius, Lithuania), ad 100 µl demineralized water.

b) Introduction of tag sequences at the C terminus

In order to simplify protein purification and make directed immobilization of P450 BM-3 possible, a C-terminal tag which was six amino acids long in each case was attached to the P450 BM-3 gene. Genomic *Bacillus megaterium* DNA acted as template. The protruding restriction ends chosen for the His, Arg and Glu tags were *Bam*HI and *Eco*RI in order subsequently to clone the modified P450-BM-3 gene into the plasmid pCYTEXP1. *Bsa*I was chosen for the 5′ and 3′ ends for the Strep tag; this makes cloning into the plasmid pASK-IBA1CA possible. *Bsa*I cuts only after the recognition sequence, resulting in the protruding restriction ends having a different sequence and thus permitting directed ligation.

The standard PCR program and the standard mixture were used for the PCRs. The differences from the standard mixture used for the His_6 tag were the primers H1 and H2, for the Arg_6 tag were the primers A1 and A2, for the Glu_6 tag were the primers G1 and G2 and for the Strep tag the primers S1 and S2.

3. Restriction cleavage, electrophoretic separation and purification of DNA fragments

DNA is cut sequence-specifically by restriction endonucleases. Restriction cleavage of DNA took place in a reaction volume of 10 µl in the presence of 2-3 µl DNA (spin-prep (Qiagen)), 1-2 U of restriction enzyme, 1 µl of restriction buffer (10x) and ad 10 µl ddH_2O by incubation of the reaction solution at 37°C (apart from *BsaI*) for two hours in accordance with the manufacturer's method. The DNA fragments were purified by agarose gel electrophoresis in accordance with the method of Sambrook et al. 1989. 0.8-2% agarose gels were used depending on the size of the DNA fragments to be fractionated. TAE buffer (40 mM Tris/HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.3) was used as electrophoresis buffer. Because of the heat evolved, a voltage of 120 V was applied for gels with an agarose concentration of up 1% and of 100 V for gels with an agarose concentration of up to 2%. Intercalating ethidium bromide (final concentration 0.25 µM) was used for visualizing the DNA in the transmitted UV light of a transilluminator (312 nm).

DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (Qiagen). This entails the DNA being immobilized by binding to a silica gel-containing column, and impurities being removed by various washing steps. Up to 8 µg of DNA (100 bp-10 kb) can be obtained by subsequent elution with demineralized water with this method.

4. Ligation and transformation into *E. coli*

The T4 DNA ligase (New England Biolabs, Beverly, USA) which catalyzes the formation of phosphodiester linkages between 5'-phosphate and 3'-hydroxy ends of the DNA, and thus connects two linear DNA molecules or makes the cyclization of a linear molecule possible, was used for ligating DNA.

For the ligation, agarose gel-purified vector and insert fragments (compare setion 3.) in the molar ratio of from 1:3 to 1:5 in favor of the insert were placed in an Eppendorf reaction vessel. Before addition of 2 µl of ligase buffer (10x) and 2-10 U of T4 DNA ligase, demineralized water was added (total volume 20 µl). The reaction mixtures were incubated at room temperature for one hour or at 7°C overnight.

To prepare competent cells for the transformation, 500 µl of an *E. coli* overnight culture were used to inoculate a 250 ml shaken flask containing 50 ml of LB medium. The culture is incubated at 37°C and 220 rpm until an OD_{600} of 0.4 - 0.6 is reached after about three hours and is then centrifuged at 5500 g and 4°C for 3 minutes. The cell pellet is resuspended in 2 ml of TSS buffer

(10 g PEG6000, 5 ml DMSO, 0.6 g $MgSO_4$, ad 100 ml LB), incubated on ice and divided into 200 µl aliquots and, after shock-freezing in liquid nitrogen, stored at -80°C.

For the transformation, 5 to 15 µl of the ligation mixture were added to 200 µl of the competent cells. After incubation on ice for 30 minutes, the transformation mixture is incubated in a Thermomixer at 42°C for 30 s, and 800 µl of LB medium at 37°C are added. The mixtures are shaken in an incubator at 37°C for one hour. The cells were then centrifuged at 5500 g (3 minutes), taken up in the drop of LB medium running back, and plated out on LB-Amp agar plates. The agar plates were incubated in an incubator at 37°C overnight.

5. DNA sequencing

The DNA sequencing was carried out using the Applied Biosystems DNA 373A automatic sequencer and the dye terminator cycle sequencing kit with AmpliTaq DNA polymerase. The Sanger dideoxy method is used for the sequencing, but dNTPs with fluorescence labels which are incorporated into the synthesized DNA fragments during the sequencing reaction are employed in place of radiolabeled dNTPs.

The reaction products were then precipitated with 100 percent ethanol cooled to -20°C. Drying of the DNA and taking up in 4 µl of a 5:1 mixture of formamide and 25 mM EDTA, pH 8.0, were followed be denaturation of each PCR mixture at 95°C for 5 minutes and immediate transfer of the samples to ice.

The DNA fragments labeled with fluorescent dyes pass through a laser beam during the gel electrophoresis. The fluorescent light emitted perpendicular to this beam is measured by photodiodes behind the gel and converted by the software into a graph.

Sequencing mixture

8 µl terminator ready reaction mix; 3.2 pmol of primer;
300-500 ng of template DNA;
H_2O ad 20 µl.

PCR program

Repetitions of the program step	Denaturation	DNA annealing	Elongation/DNA-synthesis
Once	96°C, 6 min		
25 times	96°C, 40 s	50°C, 30 s	60°C, 4 min
Once			60°C, 4 min

Composition of sequencing gel:
Urea 30 g (Roth); Rotiphorese NF 10x TBE buffer 6 ml (Roth); acrylamide/bis solution 40% (29:1) 9 ml (Roth); ddH_2O demineralized 23.5 ml.

The completed solution is filtered and degassed. The polymerization is started by adding 24 µl of TEMED and 180 µl of 10% (m/v) APS. For details of this, of the analysis of the sequence data and of the dNTP labels, reference may be made to the handbook for the automatic sequencer.

6. <u>F87A point mutation using QuikChange kit (Stratagene)</u>

To increase the sensitivity of the pNCA assay system, the amino acid phenylalanine at position 87 was replaced by an alanine. In order to be able to identify the point mutant more easily, an additional *Eae*I restriction cleavage site including the mutation position was introduced, without any other change in the amino acid sequence.

In contrast to the standard PCR program, in this case only a four-minute DNA denaturation was carried out, and the sequencing reaction step was carried out at an annealing temperature of 52.3°C, and the 17-minute elongation step was run through only 16 times. The third program was omitted.

PCR mixture:

1.2 µl of dNTP mix (200 µM), 5 µl of Pfu polymerase buffer (10x), 2.5 U of Pfu polymerase, 2.5 µl of primer F87A1 (5nM), 2.5 µl of primer F87A2 (5nM), 1 µl of pT-USC1BM3 (from mini-preparation diluted 1:20 with demineralized water), ad 50 µl demineralized water.

The "annealing" temperature of 52.3°C showed after the above PCR a sharp band with the expected fragment size on a one percent agarose gel.

After removal of the mineral oil from the reaction mixture, 1 µl of *DpnI* (10 U/µl) was added to the PCR mixture and the solution was cautiously mixed by pipetting up and down. It was briefly centrifuged and incubated at 37°C for one hour. During the digestion, the ultracompetent *E. coli* XL1-Blue were thawed on ice and distributed in 50 µl aliquots in 15 ml Falcon tubes. Addition of 1 µl of ß-mercaptoethanol and 4 µl of the digested PCR mixture and incubated on ice for 30 minutes were followed by a heat shock at 42°C for 30 s and cooling on ice for two minutes. 450 µl of warmed (oil bath temperature 42°C) NZY medium (12 g of NZamine, 5 g of yeast extract, 5 g of NaCl, pH 7.5) were added to the transformation mixture. After incubation at 37°C and 220 rpm for one hour and centrifugation (5500 g, 3 min), the cell pellet was resuspended in the remaining liquid and streaked onto LB-Amp plates.

Reference Example 4: Preparative methods

1. Cell disruption

Cell pellets with a wet biomass of up to 15 g of *E. coli* DH5α/pT-USC1BM-3 or DH5α/pT-USC1BM-3F87A were thawed on ice and suspended in 25 ml of potassium phosphate buffer (50 mM, pH 7.5, 1 mM EDTA) or Tris/HCl buffer (50 mM, pH 7.5, 1 mM EDTA). The ice-cooled *E. coli* cell suspension was disrupted by treatment with ultrasound (Branson Sonifier W250, (Dietzenbach, Germany), power output 80 W, working interval 20%) for three minutes. Before the protein purification, the cell suspension was centrifuted at 32,500 g for 20 min and filtered through a 0.22 µm Sterivex GP filter (Millipore). This clear solution is referred to as crude extract below.

2. Chromatographic purification of P450 BM-3 using anion exchangers

An ÄKTA*explorer* system (Amersham Pharmacia Biotech) with a personal computer-based OS/2-UNICORN control software v2.1 and a Frac-900 fraktion collector which permits simultaneous measurement of the total protein elution at 280 nm and P450 BM-3 elution at 417 nm was used for the protein purification.

All the analytical P450 BM-3 protein purifications by anion exchange chromatography were carried out at 200 cm/h, at room temperature and with a loading of 6-10 mg of P450 BM-3 per 100 ml of matrix. All the chromatography materials were purchased from TosoHaas (Stuttgart, Germany).

a) Packing and characterization of the chromatography columns

A P450 BM-3 purification protocol was developed by using MPD-DEAE 650S (7.5 mm x 85 mm, particle diameter 35 µm, Af=1.1, 4100 TP/m) chromatography columns and XK16/20 (16 mm x 100 mm, APB) chromatography columns packed by us and containing Toyopearl DEAE 650M (Af= 1.1, 1610 Tp/m), SuperQ 650M, (Af= 1.2, 1840 Tp/m) and QAE 550M (Af= 1.0, 1780 Tp/m) with a particle diameter of 65 µm. An INDEX200 (APB) glass column (20 cm x 19 cm, Af= 1.4, 3800 Tp/m), six liters of Toyopearl DEAE 650M and a gear pump (ISMATEC, PB, USA) were used for the preparative chromatographic purifications. In contrast to the analytical P450 BM-3 chromatographic purifications, the ÄKTA*explorer* system was used with a 1/20-divided flow as monitor, and the buffers were prepared manually before the preparative purifications. The buffers consisted of Tris/HCl (0.1 M, pH 7.8) with 1 mM EDTA plus 150 mM NaCl for the first, 250 mM NaCl for the second and 1 M NaCl for the third salt stage.

b) Choice of the pH, elution and buffer conditions

Preliminary investigations based on active measurements via NADPH consumption showed a suitable pH range between 6.8 and 8.0 for P450 BM-3 purification. Later investigations on the pH stability using the pNCA detection system confirmed this pH range. Anion exchange chromatography with linear NaCl gradients showed that a Tris/HCl buffer (0.1 M, pH 7.8) with 1 mM EDTA is distinctly superior to a phosphate buffer (0.1 M, pH 7.0 to pH 7.5) with 1 mM EDTA in relation to binding capacity and resolving ability for P450 BM-3. For these reasons, the Tris/HCl buffer system was used for all further purification optimizations.

3. Immobilization of the mutant P450 BM-3 F87A

a) Preliminary experiments on the selection of the carrier materials

Various adsorptive and covalent methods were investigated in preliminary experiments on the immobilization of P450 BM-3 F87A. 100 mg of carrier material (exception: EP-100, 50 mg) and 5 nmol of P450 BM-3 F87A (from crude extract) were used for all the preliminary experiments.

To select a suitable carrier material, for the adsorptive methods 5 nmol of P450 BM-3 F87A were added to the K_xPO_4 buffer (20 mM, pH 7.5, ad 1600 µl) carrier material suspension in a 2 ml

Eppendorf reacion vessel. The reaction vessels were attached by means of adhesive tape to the outside of a 250 ml round-bottom flask and then subjected to slow inverting rotation on a rotary evaporator for one hour for protein immobilization. To determine the adsorption capacities, the immobilisates were centrifuged at 9000 g for one minute, and 200 µl of the supernatant were removed for activity measurement.

b) Optimized immobilization procedure for DEAE 650M and Super Q650M

Up to 81 nmol of a P450 BM-3 F87A crude extract or lyophilisate can be immobilized per ml of sedimented DEAE 650M adsorbent material in 5 ml of Tris/HCl buffer (pH 7.5). With the SuperQ 650M adsorbent material it is up to 102 nmol of a P450 BM-3 F87A crude extract. For the immobilization, the lyophilisate was taken up in 4 ml of dH_2O in a 15 ml Falcon reaction vessel, and 12-pNCA was added to a final concentration of 0.1 mM. After incubation at room temperature for five minutes, 1 ml of the adsorption matrix, which had been washed twice with 5 ml of water and centrifuged (9000 g, 1 min), was subjected to inverting rotation for half an hour in analogy to the Eppendorf reaction vessels described in the previous section. After centrifugations and removal of the supernatant, the immobilisate was mixed with 5 ml of Tris/HCl buffer (pH 7.5, 0.02 mM), cautiously resuspended and centrifuged again (9000 g, 1 min). This washing process was repeated twice. Tris/HCl buffer (0.1 M, pH 7.5) was added to the immobilisate to a final volume of 2.0 ml, and the latter was resuspended and distributed in 200 µl aliquots in 1.5 ml Eppendorf reaction vessels which already contained 495 µl of this buffer. After addition of 5 µl of 12-pNCA (15 mM) and incubation for five minutes, the reaction was started in an Eppendorf shaker (Ika-Vibrax, with Janke and Kunkel VX2E attachment) by adding 100 µl of NADPH (1 mM) and was stopped after varying reaction times with 100 µl of 6 M KOH. After centrifugation at 9000 g (1 min), the supernatant was removed and the absorption at 410 nm was determined.

For the covalent immobilization of P450 BM-3 F87A using glutaraldehyde, P450 BM-3 F87A was disrupted in a 20 mM potassium phosphate buffer (pH 7.5). 5 g of amino-modified Trisopor glasses (Schueller, diameter 500-800 µm) were exposed to glutaraldehyde vapour in a closed apparatus at room temperature for between three and twelve hours. The amino-modified glasses with glutaraldehyde showed a reddish violet color which was retained even after washing twice with 100 ml of potassium carbonate solution (50 mM, pH 7.8) on a frit. From 50 to 150 mg of these

reddish violet glasses were mixed with 0.5-0.8 nmol of P450 BM-3 F87A and shaken in 2 ml Eppendorf reaction vessels in a Retsch ball mill at the lowest setting and 6°C for one hour until the solution was decolorized.

4. Enzyme membrane reactor

To check the enzyme membrane reactor concept, 25 nmol of P450 BM-3 F87A were immobilized on 4 ml of SuperQ 650M and added to 200 ml of Tris/HCl buffer (pH 7.8) in the reactor reaction vessel. After addition of 1.2 µmol of 12-pNCA at a flow rate of 40 ml/min the solution was mixed for 20 minutes without counterpressure on the filtration module. With a counterpressure of 2 psi, the reaction was started by addition of 2 ml of aqueous NADPH solution (1 mM) and the course of the reaction was followed by determination at a wavelength of 410 nm. During the course of the reaction, NADPH solution was added in 1 ml steps until conversion of 12-pNCA was complete.

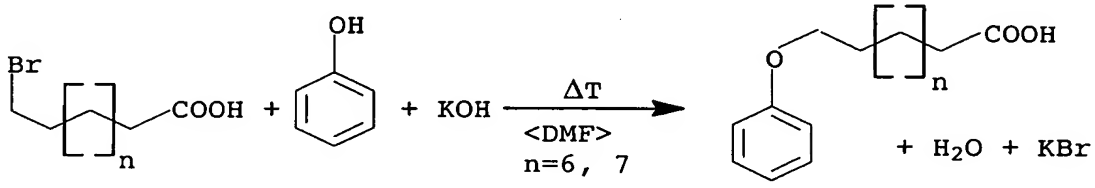
Reference Example 5: Chemical syntheses

All ^{1}H-NMR spectra were recorded with a 500 MHz NMR apparatus, and all ^{13}C-NMR were recorded with a 125 MHz NMR apparatus and $CDCl_3$ as solvent.

1. Preparation of ω-phenoxy carboxylic acids (PCA)

Reaction scheme A points to direct PCA synthesis from ω-bromo carboxylic acids.

Reaction scheme A



Mixtures

1.01 g	(3.77 mmol)	of bromoundecanoic acid or
1.05 g	(3.77 mmol)	of bromododecanoic acid
355 mg	(3.77 mmol)	of phenol
417 mg	(7.44 mmol)	of potassium hydroxide

50 ml of DMF

General reaction procedure

Phenol and potassium hydroxide are added to the bromo-fatty acid dissolved in DMF. The solution was heated under reflux at 160°C for 6-7 hours, and the reaction was followed by thin-layer chromatography (TLC, mobile phase petroleum ether : diethyl ether 1:1). After removal of the solvent, the brown precipitate was dissolved in a water-diethyl ether two-phase system (1:1), and dilute hydrochloric acid was used to adjust to a pH of 2. After extraction of the aqueous phase with diethyl ether, the solvent was removed from the combined diethyl ether fractions in a rotary evaporator, the residue was taken up in petroleum ether : ethyl acetate 1:1 and purified using a DC60 silica gel chromatography column (Merck) and the same solvent mixture.

Characterization:

ω-Phenoxydodecanoic acid (12-PCA): yield: 76%; m_p:= 98-99°C
calculated: C 73.93% H 9.65% O 16.41%, found: C 73.92% H 9.67%

^{1}H-NMR: δ:= 7.26-7.29 (m, 2H, phenyl-), 6.88-6.94 (m, 3H, phenyl), 3.94 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.35 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.74-1.80 (m, 2H, -O-CH_2-CH_2-), 1.59-1.66 (m, 2H, -CH_2-CH_2-COO), 1.42-1.48 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.29-1.35 (m, 12 H, -O-CH_2-CH_2-CH_2-$(CH_2)_6$-CH_2-).

^{13}C-NMR: δ:= 180.4 (-COO), 159.3 (C 1'), 129.8 (C 3'), 120.8 (C 4'), 114.7 (C 2'), 68.3 (-O-CH_2-), 34.5 (-CH_2-COO-), 29.9, 29.8, 29.7, 29.7, 29.5, 29.4, 29.1, 26.5, 25.1 (C-3-C-11).

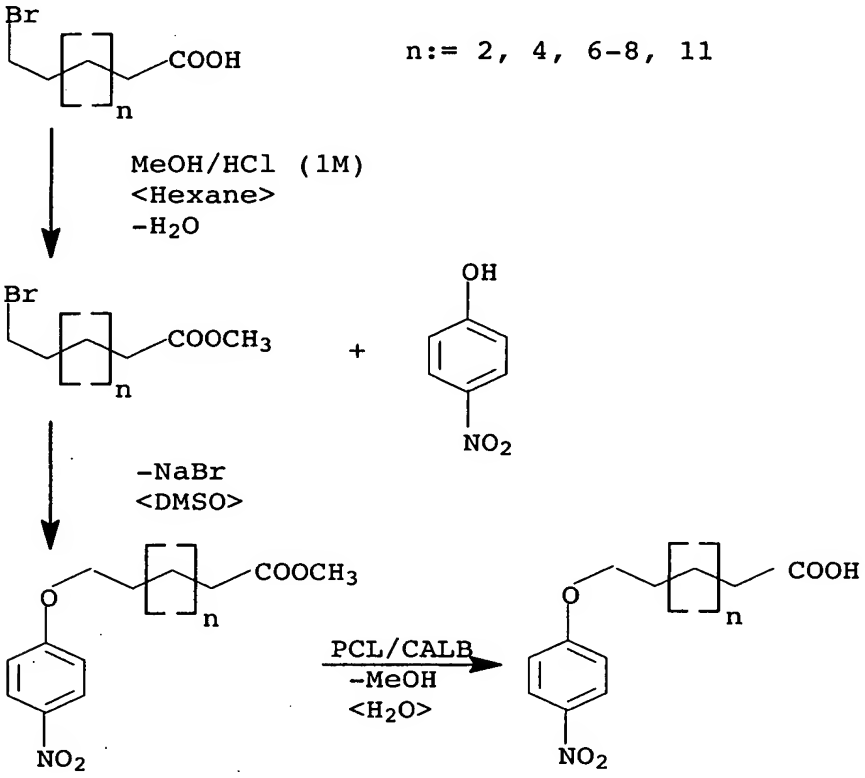
ω-Phenoxyundecanoic acid (11-PCA): yield: 78%; m_p:= 95°C,
calculated: C 73.35% H 9.41% O 17.24%, found: C 73.32% H 9.44%

^{1}H-NMR: δ:= 7.18-7.21 (m, 2H, phenyl-), 6.81-6.86 (m, 3H, phenyl), 3.87 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.27 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.67-1.73 (m, 2H, -O-CH_2-CH_2-), 1.53-1.57 (m, 2H, -CH_2-CH_2-COO), 1.35-1.39 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.18-1.30 (m, 10H, -O-CH_2-CH_2-CH_2-$(CH_2)_5$-CH_2-).

^{13}C-NMR: δ:= 180.5 (-COO), 159.5 (C 1'), 129.8 (C 3'), 120.7 (C 4'), 114.7 (C 2'), 68.2 (-O-CH_2-), 34.5 (-CH_2-COO-), 29.9, 29.7, 29.7, 29.7, 29.6, 29.4, 26.4, 25.0 (C-3-C-10).

2. Preparation of *p*-nitrophenoxy carboxylic acids (pNCA)

In contrast to the PCA syntheses, a corresponding synthesis of the pNCA compounds was not possible. pNCA synthesis was possible only after esterification of the ω-bromo carboxylic acids and anhydrous conditions were used.



General procedure for the synthesis:

Esterification of ω-bromo carboxylic acids in hexane in accordance with a standard method (Becker et al. 1993) using anhydrous methanol or ethanol solution containing 1 M HCl afforded ω-bromo carboxylic acid methyl esters and ω-bromo carboxylic acid ethyl esters. The course of the reaction was followed by TLC (mobile phase hexane : diethyl ether : acetic acid 70:30:1). The aqueous phase is extracted twice with diethyl ether, and the organic phase is washed twice with saturated sodium bicarbonate solution and once with demineralized water, dried with anhydrous sodium sulfate overnight, filtered and purified by chromatography using petroleum ether : diethyl ether in the ratio 70:30 as mobile phase. After the solvent had been evaporated off in a rotary evaporator, the esters were connected to high vacuum for one hour. In order to prepare the pNCA esters, a small excess of 18.5 nmol of p-nitrophenol was added to 17 mmol

of ω-bromo carboxylic ester dissolved in 100 ml of DMSO. The solution was heated at 120°C under a reflux condenser for two to three hours. After the pale brownish solution had been cooled to room temperature, demineralized water was added dropwise in order to precipitate the pNCA esters. The precipitate was filtered, washed with 1.5 liters of ice-cold water to remove excess p-nitrophenol and recrystallized in DMSO to give a white powder. To hydrolyze the pNCA esters, a lipase mixture consisting of 100 mg of *Pseudomonas cepacia* (PCL) and 100 mg of *Candida antarctica* lipase B from Amano (Nagoya, Japan) was added to 100 ml of an acetone/water mixture (pH 7.5, 0.2 M potassium phosphate, up to 50% acetone). After addition of the pNCA esters (5 mmol), the suspension was stirred at room temperature for up to 16 h. To remove the immobilized lipase, the clear suspension was filtered and the pNCAs were crystallized by evaporation of acetone. The pNCA compounds were washed with cold water, recrystallized in DMSO and purified by chromatography on silica gel DC60 (Merck, Darmstadt) (petroleum ether : ethyl acetate 1:1 to 1:3).

Characterization of the pNCAs

The stated yields of the pNCA are always based on the corresponding pNCA esters as precursors, and the yields of the pNCA esters are always based on the relevant ω-bromo carboxylic acids.

p-Nitrophenoxyhexanoic acid (6-pNCA): yield: 81%; m_p:= 94°C

^{1}H-NMR: δ:= 8.18 (d, 2H, NO_2-phenyl-, J= 9.3Hz), 6.94 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.06 (t, 2H, phenyl-O-CH_2-, J= 6.3 Hz), 2.42 (t, 2H, -CH_2-COO, J= 7.1 Hz), 1.80-1.92 (m, 2H, -O-CH_2-CH_2-), 1.68-1.77 (m, 2H, -CH_2-CH_2-COO), 1.49-1.61 (m, 2H, -O-CH_2-CH_2-).

^{13}C-NMR: δ:= 180.0 (-COOH), 164.1 (C-4'), 141.4 (C-1'), 125.9 (C-2'), 114.4 (C-3'), 68.5 (-O-CH_2-), 33.9 (-CH_2-COOH), 28.6, 25.5, 24.3 (C-3, C-5, C-4).

Ethyl *p*-nitrophenoxyhexanoate (6-pNCAEt): yield: 81%

^{1}H-NMR: δ:= 8.09 (d, 2H, NO_2-phenyl-, J= 9.3Hz), 6.85 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.05 (q, 2H, -COO-CH_2-), 3.97 (t, 2H, phenyl-O-CH_2-, J= 6.4 Hz), 2.26 (t, 2H, -CH_2-COO, J= 7.3 Hz), 1.69-1.82 (m, 2H, -O-CH_2-CH_2-), 1.57-1.67 (m, 2H, -CH_2-CH_2-COO), 1.40-1.50 (m, 2H, -O-CH_2-CH_2-), 1.17 (t, 3H, -COOCH_2-CH_3).

^{13}C-NMR: δ:= 173.7 (-COO), 164.3 (C 4'), 141.5 (C 1'), 126.0 (C 2'), 114.6 (C 3'), 68.7 (-O-CH_2-), 60.4 (-COO-CH_2-), 34.3 (-CH_2-COO-), 28.8, 25.7, 24.8 (C-3, C-5, C-4), 14.4 (-O-CH_2-CH_3).

p-Nitrophenoxyoctanoic acid (8-pNCA): yield: 92%; m_p:= 98°C

^{1}H-NMR: δ:= 8.11 (d, 2H, NO_2-phenyl-, J= 9.2 Hz), 6.86 (d, 2H, phenyl-O-, J= 9.3 Hz), 3.97 (t, 2H, phenyl-O-CH_2-, J= 6.4 Hz), 2.29 (t, 2H, -CH_2-COO, J= 7.4 Hz), 1.69-1.80 (m, 2H, -O-CH_2-CH_2), 1.56-1.61 (m, 2H, -CH_2-CH_2-COO), 1.32-1.43 (m, 6H, -O-CH_2-CH_2-$(CH_2)_3$-CH_2-).

^{13}C-NMR: δ:= 180.2 (-COOH), 164.3 (C 4'), 141.4 (C 1'), 125.9 (C 2'), 114.4 (C 3'), 68.8 (-O-CH_2-), 34.0 (-CH_2-COOH), 28.9, 28.9, 28.9, 25.8, 24.6 (C-3-C-7).

Methyl *p*-nitrophenoxyoctanate (8-pNCAMe): yield: 79%

^{1}H-NMR: δ:= 8.10 (d, 2H, NO_2-phenyl-, J= 9.2 Hz), 6.86 (d, 2H, phenyl-O-, J= 9,3 Hz), 3.97 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 3.59 (s, 3H, -$COOCH_3$), 2.24 (t, 2H, -CH_2-COO, J= 7.4 Hz), 1.69-1.77(m, 2H, -O-CH_2-CH_2-), 1.51-1.59 (m, 2H, -CH_2-CH_2-COO), 1.27-1.43 (m, 6H, -O-$(CH_2)_2$-$(CH_2)_3$-).

^{13}C-NMR: δ:= 174.2 (-COO), 164.3 (C 4'), 141.4 (C 1'), 125.9 (C 2'), 114.4 (C 3'), 68.8 (-O-CH_2-), 51.5 (-COO-CH_3), 34.0 (-CH_2-COO-), 29.0, 28.9, 28.9, 25.8, 24.9 (C-3-C-7).

p-Nitrophenoxydecanoic acid (10-pNCA): yield: 84%; m_p:= 101°C

^{1}H-NMR: δ:= 8.18 (d, 2H, NO_2-phenyl-, J= 9.2 Hz), 6.93 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.03 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.35 (t, 2H, -CH_2-COO, J= 7.4 Hz), 1.75-1.86 (m, 2H, -O-CH_2-CH_2-), 1.60-1.63 (m, 2H, -CH_2-CH_2-COO), 1.32-1.45 (m, 10H, -O-CH_2-CH_2-$(CH_2)_5$-CH_2-).

^{13}C-NMR: δ:= 180.4 (-COO), 164.4 (C 4'), 141.5 (C 1'), 126.1 (C 2'), 114.6 (C 3'), 69.1 (-O-CH_2-), 34.2 (-CH_2-COOH), 29.9, 29.4, 29.4, 29.3, 29.2, 26.3, 26.1, 24.8 (C-3-C-9).

Methyl *p*-nitrophenoxydecanoate (10-pNCAMe): yield: 73%

^{1}H-NMR: δ:= 8.11 (d, 2H, NO_2-phenyl-, J= 9.2 Hz), 6.86 (d, 2H, phenyl-O-, J= 9.3 Hz), 3.97 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 3.59 (s, 3H, -$COOCH_3$), 2.23 (t, 2H, -CH_2-COO, J= 7.5 Hz),

1.68-1.77 (m, 2H, $-O-CH_2-CH_2-$), 1.52-1.57 (m, 2H, $-CH_2-CH_2-COO$), 1.22-1.45 (m, 10H, $-O-CH_2-CH_2-(CH_2)_5-CH_2-$).

^{13}C-NMR: δ:= 174.2 (-COO), 164.2 (C 4'), 141.2 (C 1'), 125.8 (C 2'), 114.3 (C 3'), 68.8 ($-O-CH_2-$), 51.3 ($-COOCH_3$), 34.3 ($-CH_2-COO-$), 29.2, 29.1, 29.1, 29.0, 28.9, 25.8, 24.8 (C-3-C-9).

p-Nitrophenoxyundecanoic acid (11-pNCA): yield: 89%; m_p:= 102°C

^1H-NMR: δ:= 8.19 (d, 2H, NO_2-phenyl-, J=9.2 Hz), 6.94 (d, 2H, phenyl-O-, J= 9.1 Hz), 4.05 (t, 2H, phenyl-$O-CH_2-$, J= 6.5 Hz), 2.35 (t, 2H, $-CH_2-COO$, J= 7.6 Hz), 1.79-1.84 (m, 2H, $-O-CH_2-CH_2-$), 1.61-1.66 (m, 2H, $-CH_2-CH_2-COO$), 1.43-1.49 (m, 2H, $-O-CH_2-CH_2-CH_2-$), 1.32-1.45 (m, 10H, $-O-CH_2-CH_2-CH_2-(CH_2)_5-CH_2-$).

^{13}C-NMR: δ:= 180.12 (-COO), 164.3 (C 4'), 141.3 (C 1'), 125.9 (C 2'), 114.4 (C 3'), 68.9 ($-O-CH_2-$), 34.0 ($-CH_2-COO-$), 29.4, 29.3, 29.3, 29.2, 29.0, 29.0, 25.9, 24.6 (C-3-C-10).

Methyl *p*-nitrophenoxyundecanoate (11-pNCAMe): yield: 80%

^1H-NMR: δ:= 8.18 (d, 2H, NO_2-phenyl-, J=9.3 Hz), 6.93 (d, 2H, phenyl-O-, J= 9.2 Hz), 4.03 (t, 2H, phenyl-$O-CH_2-$, J= 6.5 Hz), 3.66 (s, 3H, $-COOCH_3$), 2.29 (t, 2H, $-CH_2-COO$, J= 7.5 Hz), 1.75-1.86 (m, 2H, $-O-CH_2-CH_2-$), 1.58-1.64 (m, 2H, $-CH_2-CH_2-COO$), 1.29-1.45 (m, 12H, $-O-CH_2-CH_2-(CH_2)_6-CH_2-$).

^{13}C-NMR: δ:= 174.5 (-COO), 164.4 (C 4'), 141.5 (C 1'), 126.1 (C 2'), 114.6 (C 3'), 69.0 ($-O-CH_2-$), 51.6 ($-COOCH_3$), 34.2 ($-CH_2-COO-$), 29.6, 29.5, 29.4, 29.4, 29.3, 29.1, 26.1, 25.1 (C-3-C-10).

p-Nitrophenoxydodecanoic acid (12-pNCA): yield: 89%; m_p:= 106°C

calculated: C 64.07% H 8.07% N 4.06%, O 23.71%, found: C 63.99% H 8.18% N 4.15%

^1H-NMR: δ:= 8.19 (d, 2H, NO_2-phenyl-, J=9.3 Hz), 6.94 (d, 2H, phenyl-O-, J= 9.4 Hz), 4.04 (t, 2H, phenyl-$O-CH_2-$, J= 6.6 Hz), 2.35 (t, 2H, $-CH_2-COO$, J= 7.5 Hz), 1.79-1.84 (m, 2H, $-O-CH_2-CH_2-$), 1.60-1.66 (m, 2H, $-CH_2-CH_2-COO$), 1.43-1.49 (m, 2H, $-O-CH_2-CH_2-CH_2-$), 1.29-1.35 (m, 12H, $-O-CH_2-CH_2-CH_2-(CH_2)_6-CH_2-$).

^{13}C-NMR: δ:= 180.4 (-COO), 164.3 (C 4'), 141.3 (C 1'), 125.9 (C 2'), 114.4 (C 3'), 68.9 ($-O-CH_2-$), 34.1 ($-CH_2-COO-$), 29.5, 29.4, 29.3, 29.2, 29.2, 29.0, 29.0, 25.9, 24.7 (C-3-C-11).

Ethyl *p*-nitrophenoxydodecanoate (12-pNCAEt): yield: 73%

^{1}H-NMR: δ:= 8.18 (d, 2H, NO_2-phenyl-, J=9.2 Hz), 6.94 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.12 (q, 2H, -COO-CH_2-, J= 7.1 Hz) 4.05 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.29 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.70-1.87 (m, 2H, -O-CH_2-CH_2-), 1.59-1.69 (m, 2H, -CH_2-CH_2-COO), 1.32-1.45 (m, 14H, -O-CH_2-CH_2-$(CH_2)_7$-CH_2-), 1.26 (t, 3H, -$COOCH_2$-CH_3, J= 7.2 Hz).

^{13}C-NMR: δ:= 174.8 (-COO), 164.4 (C 4'), 141.5 (C 1'), 126.1 (C 2'), 114.6 (C 3'), 69.0 (-O-CH_2-), 60.5 (-COO-CH_2-), 34.2 (-CH_2-COO-), 29.6, 29.5, 29.4, 29.4, 29.3, 29.2, 29.1, 26.1, 25.1 (C-3-C-11), 14.4 (-COO-CH_2-CH_3).

p-Nitrophenoxypentadecanoic acid (15-pNCA): yield: 87%; m_p:= 115°C

^{1}H-NMR: δ:= 8.19 (d, 2H, NO_2-phenyl-, J=9.2 Hz), 6.94 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.05 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.35 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.79-1.85 (m, 2H, -O-CH_2-CH_2-), 1.59-1.66 (m, 2H, -CH_2-CH_2-COO), 1.43-1.49 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.26-1.35 (m, 18H, -O-CH_2-CH_2-CH_2-$(CH_2)_9$-CH_2-).

^{13}C-NMR: δ:= 180.0 (-COO), 164.3 (C 4'), 141.3 (C 1'), 126.0 (C 2'), 114.4 (C 3'), 68.9 (-O-CH_2-), 34.0 (-CH_2-COO-), 29.6, 29.6, 29.5, 29.4, 29.4, 29.3, 29.3, 29.2, 29.1, 29.0, 25.9, 24.7 (C-3-C-14).

Methyl *p*-nitrophenoxypentadecanoate (15-pNCAMe): yield: 75%

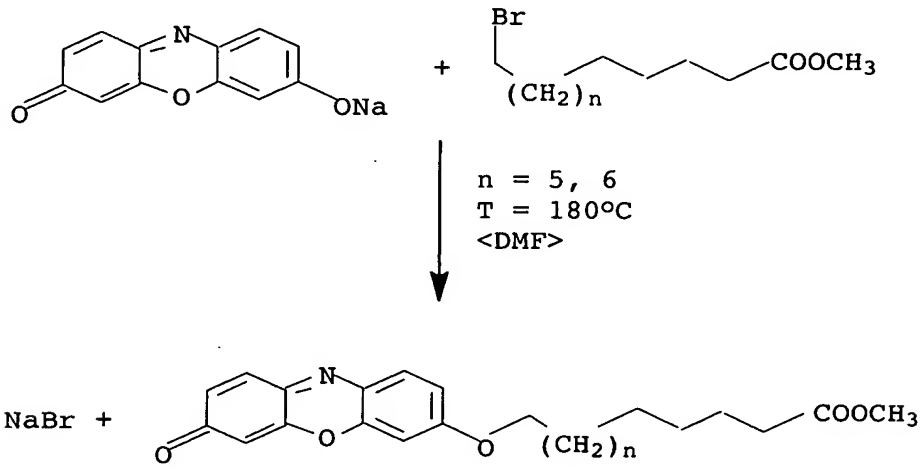
^{1}H-NMR: δ:= 8.19 (d, 2H, NO_2-phenyl-, J=9.2 Hz), 6.94 (d, 2H, phenyl-O-, J= 9.3 Hz), 4.05 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 3.66 (s, 3H, -$COOCH_3$), 2.26 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.79-1.85 (m, 2H, -O-CH_2-CH_2-), 1.59-1.66 (m, 2H, -CH_2-CH_2-COO), 1.43-1.49 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.26-1.35 (m, 18H, -O-CH_2-CH_2-CH_2-$(CH_2)_9$-CH_2-).

^{13}C-NMR: δ:= 174.6 (-COO), 164.3 (C 4'), 141.3 (C 1'), 126.0 (C 2'), 114.4 (C 3'), 68.9 (-O-CH_2-), 51.7 (-$COOCH_3$), 34.0 (-CH_2-COO-), 29.7, 29.6, 29.5, 29.5, 29.4, 29.3, 29.3, 29.2, 29.1, 29.1, 26.1, 25.0 (C-3-C-14).

3. <u>Preparation of resorufinyl carboxylic acids (RCA)</u>

a) Preparation of methyl ω-resorufinyldodecanoate and methyl ω-resorufinylundecanoate

For the RCA synthesis, the ω-bromo carboxylic acids were esterified in accordance with the procedure for the pNCA compouds, reacted with the sodium salt of resorufin in DMF and hydrolyzed using a PCL/CAL-B lipase mixture.



Mixture for methyl ω-resorufinyllaurate:

0.90 g	(3.1 mmol)	of methyl 12-bromolaurate
0.72 g	(3.1 mmol)	of resorufin
50 ml		of DMF

Mixture for methyl ω-resorufinylundecanoate:

0.75 g	(2.7 mmol)	of methyl 11-bromoundecanoate
0.63 g	(2.7 mmol)	of resorufin
50 ml		of DMF

Procedure:

Resorufin is added to a solution of the bromo-fatty acid ester in DMF but does not completely dissolve. The dark violet suspension is heated under reflux for four hours, and the reaction is followed by TLC (mobile phase petrol ether:diethyl ether 1:1). After removal of the solvent, the brown precipitate is dissolved in petroleum ether:ethyl acetate 1:1 for half an hour and purified using a DC60 (Merck) silica gel chromatography column in the same mobile phase mixture. The chosen column should not be

too large because the compound decomposes to a small extent on the column.

Methyl ω-resorufinyllaurate: yield: 17%

^{1}H-NMR: δ:= 7.62 (d, 1H, -H-9′, J= 8.89 Hz), 7.35 (d, 1H, -H-1′, J= 9.79 Hz), 6.85 (dd, 1H, -H-8′, $J_{8',9'}$= 8.87 Hz, $J_{8',6'}$ = 2.41 Hz), 6.76 (dd, 1H, -H-2′, $J_{2',1'}$= 9.74 Hz, $J_{2',4'}$= 1.74 Hz), 6.73 (d, 1H, -H-6′, J= 2.40 Hz), 6.25 (d, 1H, -H-4′, J= 1.74 Hz), 3.98 (t, 2H, phenyl-O-CH_2-, J= 6.50 Hz), 3.59 (s, 3H, -COO-CH_3), 2.23 (t, 2H, -CH_2-COO, J= 7.50 Hz), 1.73-1.79 (m, 2H, -O-CH_2-CH_2-), 1.53-1.56 (m, 2H, -CH_2-CH_2-COO), 1.37-1.41 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.22-1.29 (m, 12H, -O-CH_2-CH_2-CH_2-$(CH_2)_6$-CH_2-).

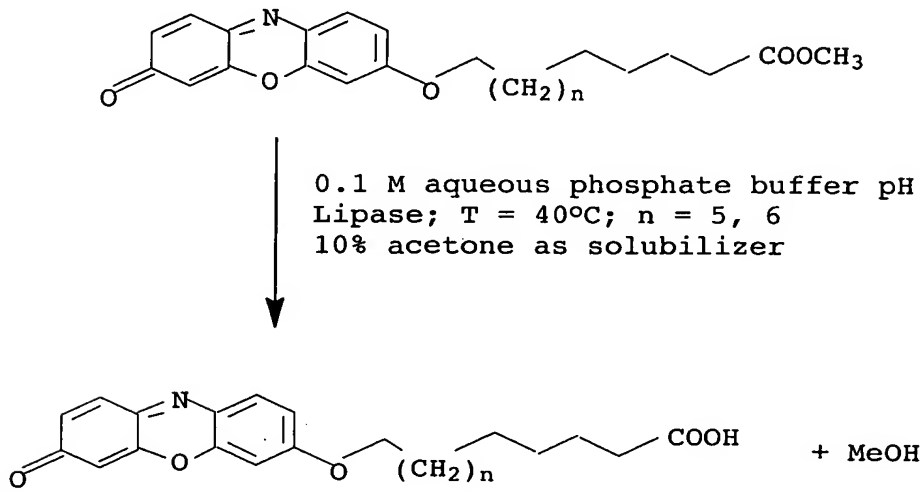
^{13}C-NMR: δ:= 186.7 (-C 3′), 174.7 (-COO), 163.7, 150.3, 146.1, 145.7 (C 4a′, C-5a′, C-9a′, C-10a′), 135.1, 134.5, 131.9, 128.7, 114.5, 107.1, 100.8 (C 2′, C 4′, C 1′, C 9′, C 6′, C 8′, C 9′), 69.5 (-O-CH_2-), 51.9 (-COO-CH_3), 34.5 (-CH_2-COO-), 30.1, 29.8, 29.7, 29.7, 29.6, 29.5, 29.3, 26.3, 25.3 (C-3-C-11).

Methyl ω-resorufinylundecanoate: yield: 15%;

^{1}H-NMR: δ:= 7.62 (d, 1H, -H-9′, J= 8.91 Hz), 7.35 (d, 1H, -H-1′, J= 9.70 Hz), 6.86 (dd, 1H, -H-8′, $J_{8',9'}$= 8.90 Hz, $J_{8',6'}$= 2.53 Hz), 6.76 (dd, 1H, -H-2′, $J_{2',1'}$= 9.72 Hz, $J_{2',4'}$= 1.95 Hz), 6.73 (d, 1H, -H-6′, J= 2.54 Hz), 6.25 (d, 1H, -H-4′, J= 1.94 Hz), 3.98 (t, 2H, phenyl-O-CH_2-, J= 6.49 Hz), 3.60 (s, 3H,-COO-CH_3), 2.23 (t, 2H, -CH_2-COO, J= 7.50 Hz), 1.74-1.79 (m, 2H, -O-CH_2-CH_2-), 1.54-1.57 (m, 2H, -CH_2-CH_2-COO), 1.35-1.42 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.18-1.29 (m, 10H, -O-CH_2-CH_2-CH_2-$(CH_2)_5$-CH_2-).

^{13}C-NMR: δ:= 186.7 (-C 3′), 174.7 (-COO), 163.7, 150.3, 146.1, 145.7 (C 4a′, C-5a′, C-9a′, C-10a′), 135.0, 135.0, 132.0, 128.6, 114.5, 107.0, 100.8 (C 2′, C 4′, C 1′, C 9′, C 6′, C 8′, C 9′), 69.5 (-O-CH_2-), 51.8 (-COO-CH_3), 34.5 (-CH_2-COO-), 29.9, 29.8, 29.7, 29.6, 29.5, 29.3, 26.3, 25.3 (C-3-C-10).

b) Preparation of ω-resorufinyldodecanoic acid and ω-resorufinylundecanoic acid



Preliminary experiments:

Chemical hydrolysis using NaOH led to cleavage of the ester, but isolation of the required yellow-orange product was possible in yields of only 10%; the cause of this is decomposition of the resorufinyl aromatic system in strongly alkaline solutions (pH>12) with loss of color. Five products were detectable on TLC plates. In alkaline medium, the resorufinyl-fatty acid ester shows a greenish color. Cleavage using lipases was considerably more successful after optimization of the workup. To choose a suitable lipase, the following lipases were tested in a 0.1 M sodium phosphate buffer pH 7.5 with 10% acetone as solubilizer at 40°C in a Thermomixer overnight: *Candida antarctica lipase A* and *B*, *Aspergillus niger* lipase and *Pseudomonas cepacia lipase* (PCL).

The *Candida antarctica lipase B* and the *PCL* cleave the resorufinyl carboxylic esters, and eventually the PCL was used for the preparative ester cleavage.

Mixture for ω-resorufinyldodecanoic acid:

40 mg	(94 μmol)	of methyl ω-resorufinyldodecanoate
2 ml		of acetone
18 ml		sodium phosphate buffer, pH 7.3
spatula tip		of PCL

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Mixture for ω-resorufinylundecanoic acid:

36 mg	(30 μmol)	of methyl ω-resorufinylundecanoate
2 ml		of acetone
18 ml		of sodium phosphate buffer, pH 7.3
spatula tip		of PCL

Procedure:

The esters are dissolved in acetone in a 50 ml round-bottom flask and, after addition of 9 ml of sodium phosphate buffer, pH 7.3, a spatula tip of PCL is added, and the mixture is incubated at 40°C overnight. The course of the reaction is followed by TLC (mobile phase petroleum ether : diethyl ether 1:1). After cautious extraction with chloroform several times, the chloroform phase is washed twice with demineralized water, dried over sodium sulfate for four hours, filtered and concentrated in a rotary evaporator.

ω-Resorufinyllauric acid: yield: 73%

^{1}H-NMR: δ:= 7.62 (d, 1H, -H-9', J= 8.9 Hz), 7.35 (d, 1H, -H-1', J= 9.8 Hz), 6.85 (dd, 1H, -H-8', $J_{8',9'}$= 8.87 Hz, $J_{8',6'}$= 2.4 Hz), 6.76 (dd, 1H, -H-2', $J_{2',1'}$= 9.7 Hz, $J_{2',4'}$= 1.7 Hz), 6.73 (d, 1H, -H-6', J= 2.4 Hz), 6.25 (d, 1H, -H-4', J= 1.7 Hz), 3.98 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.34 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.73-1.79 (m, 2H, -O-CH_2-CH_2-), 1.55-1.59 (m, 2H, -CH_2-CH_2-COO), 1.37-1.41 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.22-1.29 (m, 12H, -O-CH_2-CH_2-CH_2-$(CH_2)_6$-CH_2-).

^{13}C-NMR: δ:= 186.6 (-C 3'), 180.4 (-COO), 163.7, 150.3, 146.1, 145.7 (C 4a', C-5a', C-9a', C-10a'), 135.1, 134.5, 131.9, 128.7, 114.5, 107.1, 100.8 (C 2', C 4', C 1', C 9', C 6', C 8', C 9'), 69.5 (-O-CH_2-), 34.2 (-CH_2-COO-), 30.1, 29.8, 29.7, 29.7, 29.6, 29.5, 29.3, 26.3, 25.3 (C-3-C-11).

ω-Resorufinylundecanoic acid: yield: 78 %

^{1}H-NMR: δ:= 7.63 (d, 1H, -H-9', J= 8.9 Hz), 7.35 (d, 1H, -H-1', J= 9.7 Hz), 6.85 (dd, 1H, -H-8', $J_{8',9'}$= 8.9 Hz, $J_{8',6'}$= 2.53 Hz), 6.76 (dd, 1H, -H-2', $J_{2',1'}$= 9.7 Hz, $J_{2',4'}$= 1.9 Hz), 6.73 (d, 1H, -H-6', J= 2.5 Hz), 6.25 (d, 1H, -H-4', J= 1.9 Hz), 3.98 (t, 2H, phenyl-O-CH_2-, J= 6.5 Hz), 2.35 (t, 2H, -CH_2-COO, J= 7.5 Hz), 1.74-1.79 (m, 2H, -O-CH_2-CH_2-), 1.54-1.59 (m, 2H, -CH_2-CH_2-COO), 1.35-1.42 (m, 2H, -O-CH_2-CH_2-CH_2-), 1.18-1.29 (m, 10H, -O-CH_2-CH_2-CH_2-$(CH_2)_5$-CH_2-).

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^{13}C-NMR: δ:= 186.7 (-C 3′), 180.7 (-COO), 163.7, 150.3, 146.1, 145.7 (C 4a′, C-5a′, C-9a′, C-10a′), 135.0, 135.0, 132.0, 128.6, 114.5, 107.0, 100.8 (C 2′, C 4′, C 1′, C 9′, C 6′, C 8′, C 9′), 69.5 (-O-CH_2-), 34.1 (-CH_2-COO-), 29.9, 29.8, 29.7, 29.6, 29.5, 29.3, 26.3, 25.3 (C-3-C-10).

c) Activity test for P450 BM-3 with ω-resorufinyllauric acid and ω-resorufinylundecanoic acid:

A spatula tip of the 11- or 12-RCA was dissolved in 0.5 ml of acetone, ethanol, THF, dioxane and 5.5 ml of potassium phosphate buffer (pH 7.5, 0.1 M). 5 ml of potassium phosphate buffer (pH 7.5, 0.1 M) were added to the organic solvents acting as solubilizers. Addition of 5 nmol of P450 was followed by incubation at room temperature for 10 minutes, and the P450 BM-3 was reduced by addition of 200 μl of NADPH (50 μM). After one hour, 200 μl of the respective reaction mixtures were transferred into a microtiter plate. Corresponding blank samples without added NADPH are additionally pipetted into the second row.

Reference Example 6: Analytical methods

1. <u>Protein separation in SDS polyacrylamide gel electrophoresis (SDS-PAGE)</u>

Proteins can be fractionated according to size by SDS-PAGE. Before the gel electrophoresis, purified protein samples were mixed 1:1 with SDS sample buffer (Tris/HCl 500 mM, pH 6.8, glycerol 10% (v/v), SDS 20% (w/v), mercaptoethanol 2% (w/v), bromophenol blue 0.05%) and heated at 95°C for 5 min to denature the protein. For analysis of protein expression in *E. coli* cells, after a five-hour induction phase (OD_{578} = 1.5-2.0) 1 ml of the culture was removed by pipette and centrifuged at 12,000 g. The cell pellet was disrupted with 100 μl of the sample buffer and centrifuged at 12,000 g for 1 min. Cooling the protein samples on ice was followed by protein fractionation in electrophoresis buffer (9.0 g of Tris, 43.2 g of glycerol, 3.0 g of SDS, ddH_2O ad 600 ml) at 25 mA per gel.

A low molecular weight marker (phosphorylase B 97.4 kDa, BSA 66.3 kDa, ovalbumin 45.0 kDa, carbonic anhydrase 31.0 kDa, trypsin inhibitor 21.5 kDa, lysozyme 14.4 kDa) and a high molecular weight kit (myosin 200 kDa, β-galactosidase 116.25 kDa, phosphorylase B 97.4 kDa, BSA 66.3 kDa, ovalbumin 45 kDa) from Bio-Rad (Richmond, USA) were used as protein standard.

Used for protein separation was a 7.5 percent resolving gel (2.5 ml of Tris/HCl (1.5 M, pH 8.8), 100 µl of SDS (10% m/v), 2.5 ml of acrylamide : N,N'-methylenebisacrylamide 30 : 1 (Roth), 50 µl of APS, 5 µl of TEMED) and a 4 percent stacking gel (1 ml of stacking gel buffer (12.11 g of Tris, 0.8 g of SDS, ad H_2O 200 ml, pH 6.8 adjusted with HCl), 0.52 µl of acrylamide : N,N'-methylenebisacrylamide 30:1 (Roth), 40 µl of APS 10% (w/v), 4 µl of TEMED. Following the electrophoresis, the fractionated proteins were stained blue with staining solution (0.1% Coomassie Brilliant Blue R250, 10% (v/v) acetic acid, 30% methanol) with slow shaking for three hours. Incubation in destaining solution (10% (v/v) acetic acid, 30% methanol) took place until clear protein bands were visible. For documentation, the gel was placed free of air bubbles between a cellulose filter paper and a copying sheet and dried in a gel dryer (Bio-Rad, Model 583) at 80°C under house vacuum for two hours.

2. Determination of the protein content by detection of BCA

Protein concentrations were determined using the bicinchoninic acid (BCA) protein detection system from Pierce (St. Augustin, Germany) by spectrometry at 562 nm in accordance with the standard protocol provided by the manufacturer. Calibration lines were recorded using BSA dilutions.

3. Spectroscopic detection methods

All spectroscopic UV-Vis detections were carried out under aerobic conditions in a Pharmacia spectrophotometer, model BioChrom4060 (APB, Uppsala, Sweden), with BioChrom4060 Windows 3.11 Software v2.0.

a) Determination of the P450 concentration

P450 BM-3 concentrations were determined using the CO difference spectroscopic method (Omura et al. 1964) and an extinction coefficient of $\varepsilon = 91\ mM^{-1} * cm^{-1}$ with a chart speed of 125 nm/min at 0.1 nm intervals. For this, a spatula tip of sodium dithionite and 10 µl of a 0.1 percent (w/v) aqueous methylviologen solution were added to 5 ml of phosphate-buffered (20 mM, pH 7.3 to 7.5) P450 BM-3 crude extract. The sample, which was deep blue to bluish green depending on the P450 BM-3 concentration, was divided into two fractions. One of the two fractions was gassed with CO for one minute, avoiding foam formation, and the other is used as reference for the difference spectroscopic measurement.

b) Measurement of the reductase activity

The measurements were carried out at room temperature in 0.1 M potassium phosphate buffer pH 7.4, measuring the increase in absorption at 550 nm with $\varepsilon = 8.9\ mM^{-1}$ (Fulco et al. 1992). 1 ml of sample contained 50 nmol of cytochrome c (from horse heart), 100 nmol of NADPH and an amount of P450 BM-3 which is evident from Table 6. The same sample before addition of NADPH was used as reference. The solutions were freshly prepared and stored on ice until used the same day. To measure the reductase activity of P450 BM-3His_6, the NADPH solution was added to the cytochrome c solution after incubation for five minutes.

4. Protocols for characterization of P450 BM-3 and/or P450 BM-3 F87A

Purified P450 BM-3 F87A was used to measure the kinetic constants and the pH stability and temperature stability. Centrifuged and filtered clear crude extract was used for all the other determinations. 0.05-0.2 nmol of P450 BM-3 or P450 BM-3 F87A in a total volume of 1 ml and 8 µl of 5-15 mM pNCA solution were used per mixture for all the experiments. Characterization experiments, with the exception of the solvent tests, were carried out in 1 ml disposable plastic cuvettes. Unless described otherwise, the tests were carried out at room temperature.

a) Determination of the kinetic constants

The kinetic constants were measured after addition of 792 µl of Tris/HCl (pH 8.2, 0.2 M) and 100 µl of P450 BM-3 to 8 µl of DMSO substrate solution of varying substrate concentration. After incubation at room temperature for five minutes, the reaction was started by adding 100 µl of 1 mM NADPH solution.

b) Solvent stability of P450 BM-3 F87A

The solvent stabilities were measured in glass cuvettes (Hellma, model 6040), dissolving the substrate in acetone, dioxane, THF and ethanol instead of DMSO. The determinations were carried out by the procedure described for the kinetic measurements; however, as a difference from this, the proportion of the buffer was varied according to the amount of solvent used.

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c) pH stability and temperature stability of P450 BM-3 F87A

The measurement of the pH stability was carried out at 30°C in a Stratagene Robocycler (gradient40 model) in thin-wall 0.5 ml PCR reaction vessels (Stratagene). 392 µl of phosphate-buffered solution (50 mM, pH 4-10) were added to 0.1-0.2 nmol of P450 BM-3. After the incubation period, the P450 BM-3 solution was transferred into a cuvette, and 500 µl of Tris/HCl (0.3 M, pH 8.2) and 8 µl of 12-pNCA (6 mM) were added. After incubation for five minutes, the reaction was started by means of 100 µl of NADPH solution (1 mM). The temperature stability was investigated at a pH of 7.5 under the same conditions as the pH stability.

d) Effect of buffer salt concentrations, detergents, protease inhibitors, thio compounds, Co(III) sepulchrate on the P450 BM-3 F87A activity

The effects of these compounds on the P450 BM-3 F87A activity were investigated in analogy to the kinetic investigations, replacing a varying part of the buffer by the substance for investigation dissolved in the same buffer system.

e) Automated activity detection

Microtiter plates with 96 reaction chambers (Greiner Frickenhausen, Germany) were chosen for the automated P450 BM-3 F87A activity detection. The total reaction volume was 250 µl in a Tris/HCl buffer (0.1 M, pH 8.2) with 18 nmol of 10- and 11-pNCA, 12 nmol of 12-pNCA or 10 nmol of 15-pNCA, which were dissolved in 2.2 µl of DMSO. A Biomek2000 robotic pipettor (Beckman Instruments, Fullerton, USA) was used for the pipetting work. After an incubation time of five minutes with 0.02 nmol of P450 BM-3 F87A, the reaction was started by injecting 25 µl of an aqueous NADPH solution (1 mM) into each reaction chamber. The course of the reaction was followed by the absorption at 405 nm in a FluoStar microtiter plate reader (BMG LabTechnology, Offenburg, Germany).

f) Hydrogen peroxide investigations

The procedure for activity investigations was as described for the kinetic measurements, except that varying amounts of aqueous hydrogen peroxide were added in place of NADPH.

In the stability investigations, 0.05-0.1 nmol of P450 BM-3 F87A was incubated in the absence of pNCA substrate with varying amounts of hydrogen peroxide for five minutes. After addition of

catalase (600 U) and a further incubation time of five minutes, 60 nmol of 12-pNCA substrate were pipetted into the reaction solution. The reaction was started five minutes later by adding 100 ml of NADPH (1 mM).

g) Zn mediator investigations

The reactions were carried out in an Eppendorf reaction vessel shaker (IKA-Labortechnik Vibrax with Janke and Kunkel type VX2E attachment). The procedure for activity investigations was as described for the kinetic measurements, except that varying amounts of zinc/cobalt(III) sepulchrate were used in place of NADPH. After varying time intervals, 100 µl of sample were removed from the reaction suspension and pipetted into another 1.5 ml Eppendorf reaction vessel which contained 10 µl of KOH (6 M) to stop the reaction. After centrifugation at 12,000 g for one minute, the supernatant was removed and the absorption at 410 nm was measured.

Example 1: Cloning, expression and purification of P450 BM-3 and mutants on the gram scale

1. Cloning of the P450 BM-3 gene and of the mutant P450 BM-3 F87A and expression thereof in 30 l fermentations

The P450 BM-3 gene was isolated by PCR from the genomic DNA from *Bacillus megaterium*, provided with tags and cloned into the expression vectors pCYTEXP1 and pASK-IBA1CA as shown in Figure 1 for the pT plasmids. The sequencing primers R0_5 to L7 were used to check correct insertion of the P450 BM-3 gene, the P450 BM-3 variants provided with different tags and to check the P450 BM-3 F87A point mutant. For the PCRs by the standard protocol, the primers B1 and B2 were used for the wild-type enzyme, the primers H1 and H2 were used for the P450 BM-3His_6, the primers G1 and G2 were used for P450 BM-3Glu_6, the primers A1 and A2 were used for P450 BM-3Arg_6, and the primers S1 and S2 were used for 450 BM-3Strep. Sequence analysis revealed no mutations at all for one of in each case three sequenced pT-USC0BM3 and pT-USC1BM3 plasmids. Correct insertion of the tag was checked for pT-USC2BM3 and pT-USC3BM3 only with the primer R7. The sequenced pA-USC4BM3 clone showed two mutations in the reductase portion of the P450 BM-3 protein. The expression rate of the P450 BM-3 provided with a His_6 tag in DH5α was 300 nmol per liter of fermenter broth which was about 20% higher than for the wild type. Because of this higher expression rate, pT-USC1BM3 underwent point mutation by replacing a phenylalanine at position 87 by alanine using the Stragene QuikChange kit to increase the sensitivity of the pNCA

test. The PCR with the primers F87A1 and F87A2 results in pT-USC1BM3F87A containing at the mutation position, without a further alteration in the protein sequence, an additional *Eae*I restriction cleavage site which was used to select the clone selected for the sequencing. This additional restriction cleavage site results, after restriction cleavage with *Eae*I, in the appearance of a new band at about 800 bp in lane 2, and a distinctly smaller band, compared with the wild-type digestion, at 1.7 kbp in lane 1. After plasmid isolation, eight clones were digested with *Eae*I (2 µl of *Eae*I buffer (10x), 1 µl of *Eae*I, 3.2 µl of pT-USC1BM3, ad 20 µl ddH_2O, 1 h at 37°C). One clone was sequenced. The pT-USC1BM3F87A plasmid has an additional mutation R470C at position 470 in the linker region. No back-mutation was carried out because the R470C mutation was located at the end of the linker region which connects the P450 portion of the P450 BM-3 to the reductase portion and shows no effect at all on the P450 BM-3 activity.

2. Purification of P450 BM-3 and P450 BM-3 F87A

The aim was to develop a method for purifying P450 BM-3 and P450 BM-3 F87A which is inexpensive, quick and capable of being carried out on the preparation scale. To this end, an Arg_6, His_6, Glu_6 and Strep tag was attached to the C-terminal end of P450 BM-3. It was intended to develop affinity purifications on the basis of the His_6 and Strep tags and chromatographic purifications with ion exchangers on the basis of the Arg_6 and Glu_6 tags. An introduction of one tag at the N-terminal end of the P450 BM-3 gene was not taken into consideration because of the location of the N-terminus is in the region of the flexible substrate entry channel. The modification in this region would very probably lead to an alteration of P450 BM-3 activity and selectivity.

2.1 Protein purifications by affinity chromatography

a) P450 BM-3 with His_6 tag

In the purification of P450 BM-3His_6 by metal chelate chromatography, more than 95% of the loaded 4 mg of P450 BM-3 do not bind to the 20 ml XK16/20 affinity column with a linear 0-0.5 molar imidazole gradient (10CV). Chromatographic purification with Zn^{2+} ions was carried out by the standard Pharmacia protocol. The little purified P450 BM-3 no longer showed any activity in the NADPH assay. The cause of this inhibition might be attributed to the effect of imidazole. Imidazole brings about detachment of the fifth cysteinate ligand at the catalytic heme site. This dissociation of the fifth ligand off from the heme system leads

to immediate inactivation of P450 and is evident in the CO difference spectrum through a characteristic shift in the absorption maximum from 448 nm to 420 nm.

b) *P450 BM-3 with Strep tag*

8.2 mg of P450 BM-3Strep were used for purification. Comparable with the results for the His_6 tag purifications, P450 BM-3Strep showed no binding to the streptavidin affinity matrix on purification in accordance with the READY TO USE KIT (IBA, Göttingen).

c) P450 BM-3 with Glu_6 tag

Comparison of the linear 0-1 M NaCl gradients (12 CV, Tris/HCl buffer (0.1 M, pH 8.0)) showed no differences from the wild-type enzyme in the elution characteristics for P450 BM-3Glu_6 on use of Super 650M and DEAE 650M anion exchangers.

d) Conclusions

The results of the purification of P450 BM-3 with His_6, Glu_6 and Strep tags lead to the conclusion that the C-terminal end of P450 BM-3 is not freely accessible on the protein surface and thus cannot be used for purification purposes. In addition, in the case of the His_6 tag, elution with imidazole caused rapid protein inactivation due to displacement of the fifth ligand on the porphyrin system.

2.2 <u>Anion exchange chromatography</u>

In order to minimize the costs, it was intended to develop an efficient and time-saving purification method at reasonable cost and allowing simple scale-up for P450 BM-3. Thus, after the results of the purification of P450 BM-3 with tags, the only methods available for P450 BM-3 purification were HIC materials and ion exchangers. The decision was made in favor of anion exchangers which are usually cheaper and simpler to handle.

a) Linear and stepped gradient

In order to develop an anion exchange chromatography method, four anion exchange materials of different strengths, DEAE 650S (35 µm), DEAE 650M (65 µm), SuperQ 650M (65 µm) and QAE 550M (65 µm), were investigated for their suitability. In the initial experiments, a linear 0-1 M NaCl gradient 10 column volumes (CV) long was taken up in a Tris/HCl buffer (0.1 M, pH 7.8) with each

column material for purifying 6-10 mg of P450 BM-3 crude extract. The yield was 80-84% in all cases. The highest proportions of protein in the P450 BM-3 elution peak were shown by the DEAE material, closely followed by the SuperQ and QAE material.

For further improvement of the resolution using stepped salt gradients, the ability of the ÄKTA*explorer* system to record simultaneously the conductivity and absorption at 280 nm and 417 nm was helpful. An optimized two-step salt gradient for the DEAE 650M chromatography column has a first step of 150 mM NaCl and a second with 250 mM NaCl. Elution of P450 BM-3 starts, as is evident from the conductivities of the linear NaCl salt gradient, when the NaCl concentration is increased from 150 mM to 170 mM. The minimum upper salt step was found to be 250 mM NaCl; NaCl concentrations < 230 mM cause broadening of the P450 BM-3 elution peak, and NaCl concentrations > 300 mM result in a distinctly less good purification effect. The salt steps were optimized in a comparable way for the SuperQ 650M and QAE 550M chromatography materials.

Comparison of the linear DEAE 650M with the stepped NaCl gradient leads to an increase in the P450 BM-3 protein content in the elution peak, from 39% with the linear gradient to 84% with the stepped gradient. Comparable results were achieved with the SuperQ 650M chromatography material; the QAE 550M chromatography material showed distinctly less good purification results. The yields in all cases with the stepped purification methods were > 78%; the highest yields of 93% of P450 BM-3 in the elution peak were obtained with DEAE 650S.

Example 2: Development of a spectroscopic activity detection for P450 BM-3 and P450 BM-3 F87A

The aim was to develop a spectrometric or fluorimetric activity detection for P450 BM-3. Such an activity detection can, in contrast to standard methods such as HPLC or GC, be automated simply and thus be used for finding other P450 BM-3 enzyme variants.

In order to develop a novel activity detection for P450 BM-3, various compounds which carry the chromophore at the terminal C atom of the carboxylic acids were synthesized and investigated for their suitability.

Studies by Oliver et al. Biochemistry (1997), 36(7):1567 showed a shift in the hydroxylation profile by P450 BM-3 for lauric acid and myristic acid as substrate from subterminal positions to the

terminal position if phenylalanine at position 87 was replaced by alanine. To increase the sensitivity of the pNCA detection method, this point mutation was introduced as described in material and methods and confirmed by sequence analysis.

1. Detection principle

Figure 2 shows the principle of a spectrometric activity detection for terminally hydroxylating fatty acid hydroxylases. After terminal hydroxylation there is formation of an unstable hemiacetal which dissociates into the ω-oxy carboxylic acid and the chromophore which can be detected spectrometrically.

2. Detection of P450 BM-3 F87A activity with PCA

a) Synthesis of the phenoxy carboxylic acids (PCA)

The PCA compounds were synthesized in a one-stage synthesis as described in Reference Example 5 starting from ω-bromo carboxylic acids. Overall yields between 76 and 78% were obtained in the syntheses. The PCAs were characterized by ^{1}H-NMR, ^{13}C-NMR and melting point determination.

b) Choice of the reaction conditions

In order to be able to detect phenol formation by spectroscopy, it is obvious to measure the absorption in the wavelength range between 250 and 280 nm (Luchter-Wasylewska 1996). The low extinction coefficient at the absorption maximum at 274 nm of $\varepsilon = 1090\ M^{-1}cm^{-1}$, the difference in the absorption characteristics of NADPH and $NADP^{+}$ in this wavelength range and the formation of hydrogen peroxide in uncoupled reactions prevented continuous spectroscopic measurement of phenol formation, however. For these reasons, the Merck phenol detection kit was used to quantify product formation. The conversion rates on use of the P450 BM-3 mutant F87A with NADPH as cofactor were found to be 640 eq/min for 12-PCA and 410 eq/min for 11-PCA.

3. Detection of P450 BM-3 and P450 BM-3 F87A activity with pNCA substrates

a) Synthesis of the p-nitrophenoxy carboxylic acids (pNCA)

The pNCA compounds were synthesized in a novel, three-stage synthesis as described in Reference Example 5 starting from ω-bromo carboxylic acids by esterification, subsequent S_n2 reaction with sodium *p*-nitrophenolate and lipase-catalyzed

hydrolysis of the esters. The overall yields in the three-stage pNCA syntheses were between 61 and 69%. The pNCAs were characterized by ^{1}H-NMR, ^{13}C-NMR and melting point determination.

b) Choice of the reaction conditions

Figure 2 describes the reaction principle for the conversion of pNCA substrates by P450 BM-3 and P450 BM-3 F87A. For the activity measurements it was necessary to optimize the pH and wavelength of the spectroscopic detection system. Only deprotonated *p*-nitrophenolate (pKa 7.1) contributes to the yellow color; thus a pH >9.1 (>99% deprotonated) would be advisable for the sensitivity of detection. The pNCA compounds are stable in strongly alkaline solutions, in contrast to the P450 BM-3 protein which loses 80% of its activity within 5 min at pH 10 and 30°C. A pH of 8.1 to 8.2 was chosen as a compromise between protein stability and sensitivity of the detection method. Calculations based on the Henderson-Hasselbalch equation show that in this pH range 90-92% of the converted *p*-nitrophenol are deprotonated and contribute to the yellow color. The absorption maximum for *p*-nitrophenolate is at 400 nm. However, the heme site and the cofactor NADPH also absorb at this wavelength. P450 BM-3 has strong absorption characteristics between 350 and 450 nm which vary greatly as a function of substrate binding and the oxidation state of the iron in the catalytic site. The absorption spectrum of NADPH limits the wavelength range to >390 nm. The wavelength of 410 nm was eventually selected for the activity measurement on the basis of the reproducibility. If the sensitivity of the detection system is inadequate, wavelengths in the region of 400 nm should be used. The extinction coefficient was determined under the chosen detection conditions (pH 8.2, 410 nm) to be $\varepsilon = 13{,}200\ M^{-1}cm^{-1}$.

pNCAs have only low solubility in water so that a solubilizer is required. DMSO can be added for this purpose without influencing the P450 BM-3 F87A activity up to concentrations of 1% (v/v). 0.8% (v/v) DMSO was therefore used as solubilizer in the activity detection system.

The time required by the pNCA detection system depends greatly on the amount of enzyme used. If nmol amounts of P450 BM-3 F87A were employed in the pNCA activity detection, 1 min was sufficient for activity detection.

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c) Automation of the pNCA detection

For use in an HTS environment, the pNCA activity detection was automated using a Biomek2000 workstation and a BMG FluoStar microtiter plate reader. The reproducibility of the assay system was investigated using P450 BM-3 and the mutant F87A in 16 measurements in each case with the pNCA substrates 10-, 11-, 12- and 15-pNCA. There were differences by a factor of two to four in the total absorptions because of pipetting inaccuracies, but the differences in reactivity varied only between 2 and 6%. The best reproducibility was achieved for the P450 BM-3 F87A mutant, with deviations of ~ 2%, and the worst were achieved for the wild type and 15-pNCA, with deviations of up to 6%, from the mean of all the measurements. The causes of the differences in accuracy are the differences in the solubility of the pNCA substrates and different conversions depending on the chain length.

The F87A mutant converts 12-pNCA completely, whereas the wild-type enzyme stops after 33%. The cause is presumably the subterminally hydroxylated 12-pNCA compounds which are no longer converted or are converted further only slowly. For P450 BM-3 and P450 BM-3 F87A, the pNCA chain-length profile and the conversion from ω-hydroxylation of the pNCAs, calculated from the ratios of the observed to the measured yellow coloration of the solution, are summarized in Tables 4 and 5 below. In addition, the kinetic data were found from the Lineweaver-Burk diagrams for P450 F87A.

Table 4

Chain length of the pNCA compound	P450 BM-3 F87A k_{cat} [eq[a]/min]	P450 BM-3 F87A ω-hydroxylation products [%]	P450 BM-3 Wild type k_{cat} [eq[a]/min]	P450 BM-3 Wild type ω-hydroxylation products [%]
6	not converted	not converted	not converted	not converted
8	1	<1	2	<1
10	426	94	322	98
11	204	64	267	63
12	574	100	114	33
15	680	61	246	35

a Equivalent (eq) is used in place of the term nmol (substrate)/nmol (P450)

Table 5

Chain length of the pNCA compound	P450 BM-3 F87A k_{cat} [eq[a]/min]	K_m [mM]	k_{cat}/K_m [$M^{-1}*min^{-1}$]
10	426 32	42 8	$1.0*10^7$
11	204 18	69 7	$3.4*10^5$
12	574 47	6.2 0.3	$1.1*10^4$
15	680	-	-

a Equivalent (eq) is used in place of the term nmol (substrate)/nmol (P450)

Example 3: Novel cofactor system for P450 BM-3 and P450 BM-3 F87A

A major impediment to the industrial use of P450 systems is the costs caused by the cofactor NADPH. To solve the cost problem, an alternative cofactor concept in which NADPH has been replaced by zinc and the mediator cobalt(III) sepulchrate has been developed; zinc serves as source of electrons and cobalt(III) sepulchrate serves as electron transport system from zinc to P450 BM-3.

1. Detection of the reduction of cobalt(III) sepulchrate by zinc

Under standard conditions, cobalt(III) sepulchrate has a normal potential of -0.54 V against a calomel electrode (Creaser et al., J. Am. Chem. Soc. (1977) 99: 3181). It can, as shown in Figure 3, be reduced by Zn dust in a Tris/HCl buffer (0.2 M, pH 8.2) within seconds. In the presence of atmospheric oxygen in aqueous solutions it is oxidized back to cobalt(III) sepulchrate within a few minutes.

2. Detection of P450 BM-3 F87A activity with alternative cofactor system

2.1 pNCA assay

Figure 4 shows the conversion of 12-pNCA by the Zn/cobalt(III) sepulchrate/P450 BM-3 F87A system. With a maximum absorption of 0.8 after 8 min, the Zn/cobalt(III) sepulchrate/P450 BM-3 F87A system shows a yellow color corresponding to 70% conversion. The yellow color caused by *p*-nitrophenolate disappears completely within 20 min; the residual absorption of 0.15 derives from the mediator. Control experiments showed that Zn powder reduces the nitro group of *p*-nitrophenolate in Tris/HCl buffer (pH 8.2,

50 mM, 0.25 M KCl). Further control experiments showed that hydrogen peroxide has no oxidative effect on *p*-nitrophenolate and pNCAs.

Figure 5 depicts the described electron transfer pathways from Zn via the mediator to P450 BM-3 and the substrate conversion taking account of the shortened hydrogen peroxide reaction pathway (shunt pathway).

2.2 PCA assay

Because of the reduction of *p*-nitrophenol by zinc in a Tris/HCl buffer, 12-pNCA was replaced by 12-PCA. The phenol was detected by a Merck phenol detection kit at 495 nm. A direct photometric phenol detection at 274 nm is not possible owing to the absorption characteristics of the oxidized and reduced cobalt(III) sepulchrate. Figure 6 illustrates the slow decolorization of 12-PCA as substrate compared with 12-pNCA (Figure 4). It was possible to identify the cause of the decrease in absorption as hydrogen peroxide which is formed by reduced cobalt(II) sepulchrate in side reactions by reduction of atmospheric oxygen in aqueous solutions. In addition, hydrogen peroxide formation always occurs as product of side reactions with P450 systems as a result of uncoupled reactions. For the latter reason, further optimization of the PCA reaction conditions was not pursued further.

In the presence of 600 U of catalase, as shown in Figure 6, the decrease in absorption at 495 nm takes place distinctly more slowly.

3.1 Buffer composition

In order to avoid decolorization of the solution through reduction of *p*-nitrophenolate by zinc, initially, without success, the KCl concentration and the pH were varied within the scope of the stability of P450 BM-3 F87A (6.5-8.5). The reduction of *p*-nitrophenol by zinc can be successfully prevented, as shown in Figure 7, by additions of potassium phosphate buffer. There is reduction of *p*-nitrophenol by Zn to only a small extent ($\leq$ 10%) with a potassium phosphate content $\geq$ 10%, and with a 1:1 mixture there is no further reduction detectable even after 14 h (not depicted). As shown in Figure 7, there is initially a marked reduction of about ~25% in the content of reduced Co(II) sepulchrate in the mixing range up to 20% potassium phosphate, and it then remains constant between 60 and 70% with a potassium phosphate content between 20 and 70% (v/v). The lower

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availability, compared with the Tris/HCl buffer system, of reduced cobalt(II) sepulchrate leads to a loss of about 15% of the activity.

From the above results, a 1:1 mixture of the two buffer solutions was used for further optimizations.

3.2 Effect of the mediator concentration on the conversion and the P450 BM-3 F87A activity

Figure 8A shows the effect of the cobalt(III) sepulchrate mediator on the P450 BM-3 activity in the NADPH assay with varying mediator concentrations. At cobalt(III) sepulchrate concentrations $\geq$ 5 mM there was formation of a colloidal precipitate during the course of the reaction, and this was removed by centrifugation before the measurement of absorption. An optimal cobalt(III) sepulchrate concentration in the range 0.5-0.75 mM per 0.072 nmol of P450 BM-3 F87A is present for conversions with the zinc/cobalt(III) sepulchrate system. As shown in Figure 8B, cobalt(III) sepulchrate in concentrations up to $\leq$ 0.5 mM inhibits P450 BM-3 F87A only slightly, even in the absence of substrate. The degree of inhibition remains constant with varying incubation times and depends only on the ratio of the cobalt(III) sepulchrate and P450 BM-3 F87A concentrations.

3.3 Zn concentration

The dependence of the conversion of 0.072 nmol of P450 BM-3 F87A on the amount of zinc powder employed is shown in Figure 9. An optimal conversion was achieved in the range from 20 to 40 mg of zinc per milliliter of reaction solution. Presumably a decrease in oxygen concentration in the reaction solution due to increasing mediator-catalyzed hydrogen peroxide formation was responsible for the declines in conversion with 50 and 100 mg.

Replacement of zinc powder by zinc granules led to only very low conversion rates (factor >20 lower) even on use of amounts 100 times higher.

3.4 Effect of the H_2O_2 concentration

In order to determine the amount of the contribution of hydrogen peroxide via the 'shunt' reaction pathway to the conversion of 12-pNCA by P450 BM-3 F87A, 12-pNCA and P450 BM-3 F87A were incubated with varying hydrogen peroxide concentrations. In the range 8-20 µM hydrogen peroxide with comparable P450 BM-3 F87A activities a plateau is reached at 20-25% conversion,

irrespective of the hydrogen peroxide concentration, after one minute. Further addition of NADPH in excess (100 μM) shows that P450 BM-3 F87A is not completely inactivated by hydrogen peroxide. The residual activities are high at low hydrogen peroxide concentration and decrease with increasing hydrogen peroxide concentration (not shown).

In order to determine the stability of P450 BM-3 toward hydrogen peroxide, P450 BM-3 F87A was incubated with varying amounts of hydrogen peroxide in the absence of substrate (12-pNCA) for 5 min. After addition of catalase (600 U) and 60 nmol of substrate, the conversion rates were determined using NADPH as electron donor.

As shown in Figure 10, in the absence of substrate, even hydrogen peroxide concentrations in the 5 μM range distinctly inhibit the P450 BM-3 F87A activity. The consequence which emerges from this for a P450 BM-3 F87A enzyme membrane reactor is to operate at substrate concentrations which are as high as possible and to minimize the hydrogen peroxide formation by addition of catalase or antioxidant.

Example 4: Activity assay with further P450 BM-3 mutants

Further BM-3 mutants and the wild-type enzyme (WT) were assayed for activity on pNCA derivatives of varying chain length in analogy to Example 2. The results are summarized in Table 6 below:

Table 6

Specific activity of some P450 BM-3 mutants for pNCAs of varying chain length

	WT	F87A	L188K	V26T	R47F	S72G	A74G	M354T
15-pNCA	405[1]	410	288	519	258	439	474	560
12-pNCA	141	284	316	555	233	596	517	480
10-pNCA	339	92	207	106	52	150	103	171
8-pNCA	15	2	69	16	13	3	6	4

[1] Specific activity: nmol/min/nmol P450.

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